IMPACT OF TELOMERASE ACTIVITY AND TOLL-LIKE RECEPTOR 9 ACTIVATION ON EPSTEIN-BARR VIRUS INFECTION IN EPITHELIAL CELLS

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Jürgen Rac

aus

Deutschland

Promotionskomitee

Prof. Dr. David Nadal (Vorsitz und Leitung der Dissertation)

Dr. Michele Bernasconi

Prof. Dr. Urs F. Greber

Prof. Dr. Michael O. Hottiger

Prof. Dr. Roberto F. Speck

Dr. Bernd Wollscheid

Zürich, 2014

Meiner Familie

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The experimental work presented in this thesis was performed at the Division of Experimental Infectious Diseases and Cancer Research at the Children's University Hospital Zurich. The supervision of this thesis was conducted by Prof. Dr. David Nadal (Division of Experimental Infectious Diseases and Cancer Research, Children's University Hospital Zurich), Prof. Dr. Urs F. Greber (Institute of Molecular Life Science, University of Zurich), Prof. Dr. Michael O. Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich), Prof. Dr. Roberto F. Speck (Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich), Dr. Bernd Wollscheid (Institute of Molecular Systems Biology, ETH Zurich) and Dr. Michele Bernasconi (Division of Experimental Infectious Diseases and Cancer Research, Children's University Hospital Zurich).

The only aids used for composing this dissertation are those stated therein.

Zurich, February 2014

Jürgen Rac

I SUMMARY

Epstein-Barr virus (EBV) is one of eight known human herpesviruses and belongs to the γ-herpesvirus subfamily. EBV is extremely successful by infecting >90% of the adult human population. Most of its success can be attributed to its default state of infection, called latency. Latent EBV infection is characterized by very limited viral gene expression patterns, resulting in a maximum level of immune evasion. Latent EBV infection is tightly associated with various lymphoid and epithelial cell malignancies such as Burkitt's lymphoma and nasopharyngeal carcinoma. EBV is transmitted via saliva to the next susceptible host and establishes a life long persistent infection within the host´s B cell pool. Since EBV is an orally transmitted virus, the pharyngeal cavities are the portal of entry and exit for EBV. Thus, the epithelium, lining the pharyngeal, cavities is an important barrier that EBV has to overcome to complete its life cycle.

Within this thesis, I investigated different aspects of the EBV infection in epithelial cells. I assessed the impact of increased telomerase activity, achieved by ectopic expression of hTERT and the impact of Toll-like receptor (TLR) 9 activation on the EBV infection in epithelial cells. Increased telomerase activity is characteristic for stem and progenitor cells within the basal layers of epithelial tissues and a hallmark of cancer in general. It was shown that increased telomerase activity facilitates the establishment of an EBV infection in epithelial cells by promoting expression of latent EBV genes and therefore contributing to EBV maintenance in epithelial cells. TLRs are part of the innate immune system and are the first line of defense against invading microorganisms. There was no significant impact of TLR9 activation on the EBV infection in the employed epithelial model cell lines observed. Nevertheless, our data contributes to the hypothesis that TLR activation in epithelial cells might trigger or support lytic EBV reactivation and thus facilitating EBV transmission.

In summary, the results of this work suggest that hTERT might facilitate an EBV-driven malignant transformation of epithelial cells and indicate that EBV might actually benefit from an activated innate immune system in epithelial cells. In conclusion, this data emphasizes the need for further evaluation of hTERT expression/activity inhibitors and TLR agonists as potential treatment strategies to fight EBV-associated epithelial malignancies.

II ZUSAMMENFASSUNG

Epstein-Barr-Virus (EBV) ist eines von acht bekannten humanen Herpesviren und gehört zur Unterfamilie der γ-Herpesviren. Die Tatsache, dass >90 % der erwachsenen Bevölkerung mit dem Virus infiziert ist, macht EBV zu einem außerordentlich erfolgreichen Virus. Den größten Anteil am Erfolg von EBV kann der so genannten Latenz, dem Standard Zustand der Infektion, zugeschrieben werden. Eine latente EBV-Infektion ist gekennzeichnet durch sehr limitierte Expressionsmuster viraler Gene was zu einem Höchstmaß an Immunevasion führt und eng mit verschiedenen bösartigen B-Zell- und Epithelzell-Tumoren, wie dem Burkitt-Lymphom und dem Nasophyrynxkarzinom, verknüpft ist. EBV wird durch Speichel auf den nächsten Wirt übertragen und etabliert dann eine lebenslang persistierende Infektion innerhalb des B-Zell-Pools des Wirtes. Da EBV oral übertragen wird, stellt der Rachenraum das Ein- und Austrittsportal für EBV dar. Somit ist das Epithel des Rachenraums eine wichtige Barriere für EBV, die es für das Virus zu überwinden gilt um dessen Lebenszyklus zu vervollständigen.

Im Rahmen dieser Arbeit untersuchte ich verschiedene Aspekte der EBV-Infektion in Epithelzellen. Ich überprüfte die Auswirkungen einer erhöhten Telomerase-Aktivität, erzielt durch die ektopische Expression von hTERT, und die Wirkung von Toll-like Rezeptor (TLR) 9-Aktivierung auf die EBV-Infektion von Epithelzellen. Gesteigerte Telomerase-Aktivität ist charakteristisch für Stamm- und Vorläuferzellen in den basalen Schichten von Epithelgeweben und im Allgemeinen ein Merkmal von Krebs. Es zeigte sich, dass eine erhöhte Aktivität der Telomerase die Etablierung einer EBV-Infektion in Epithelzellen, durch Förderung der EBV Latenz-Genexpression, erleichtert und somit zur Erhaltung von EBV in Epithelzellen beiträgt. TLRs gehören zum angeborenen Immunsystem und sind somit Teil der ersten Verteidigungslinie gegen eindringende Mikroorganismen. Bei der Untersuchung der Modell Epithelzelllinien konnte ich keinen signifikanten Einfluss der Aktivierung von TLR9 auf die EBV-Infektion feststellen. Dennoch tragen unsere Daten zur Hypothese bei, dass TLR-Aktivierung in Epithelzellen eine lytische EBV-Reaktivierung auslösen oder unterstützen könnte und somit die Übertragung von EBV erleichtert.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass hTERT eine EBV-getriebene, maligne Transformation von Epithelzellen fördern könnte und deuten darauf hin, dass EBV tatsächlich von einem aktivierten, angeborenen Immunsystem in Epithelzellen

profitieren könnte. Abschließend unterstreichen diese Daten die Notwendigkeit weiterer, grundlegender Untersuchungen zur Eignung von hTERT Expressions-/Aktivitäts-Inhibitoren und TLR-Agonisten als mögliche Behandlungsstrategien zur Bekämpfung EBV-assoziierter Epithelzell-Tumoren.

III INTRODUCTION

III.1 Epstein-Barr virus

Epstein-Barr virus (EBV) is one of eight members of the *herpesviridea* family that commonly infect humans. EBV belongs to the subfamily of γ-herpesviruses and its tissue tropism *in vivo* is mainly restricted to B lymphocytes and epithelial cells (Table 1). EBV is an enveloped virus with a linear, double-stranded DNA genome of about 184 kbp (1). The virus is usually transmitted *via* saliva and is enormously successful in infecting humans since more than 90% of the adult human population is seropositive for immunoglobulin G against the viral capsid antigen (VCA) complex of EBV (2, 3). In developing countries, primary infection by EBV occurs early during childhood and the course of infection is usually asymptomatic or displays unspecific symptoms. If seroconversion is delayed to adolescence or adulthood more than 70% of the patients develop the most frequent clinical manifestation of a primary EBV infection, called infectious mononucleosis (IM) or kissing disease (4). IM is a self-limiting disease that goes along with typical clinical symptoms, such as pharyngitis, fever, fatigue, cervical lymphadenopathy, hepatosplenomegaly and T cell lymphocytosis. Like all herpesviruses, EBV can infect cells in a latent or a lytic form (1, 5). Upon primary infection, EBV persists life long in latent state within the host memory B cell pool (6). The switch from latent to lytic infection is tightly controlled by various mechanisms (7) and is under investigation as potential treatment strategy against EBV-associated malignancies. The latent EBV infection is characterized by very restricted gene expression patterns, thus leading to a maximum level of immune evasion (5, 8–11). By contrast, lytic EBV replication, or so-called productive infection, results in generation of infectious progeny virus particles and is therefore important for virus transmission from cell to cell and host to host (1, 5).

Table 1: Human herpesvirus Taxonomy, nomenclature and main tissue tropisms (adapted from (12)).

Trivial name (and acronym)	Formal name (and acronym)	Herpesvirus subfamily; genus	Primary target cells	Main site of latency
Herpes simplex virus-1 (HSV-1)	Human herpesvirus 1 (HHV-1)	Alpha; Simplexvirus	Mucoepithelia	Sensory and cranial nerve ganglia
Herpes simplex virus-2 (HSV-2)	Human herpesvirus 2 (HHV-2)	Alpha; Simplexvirus	Mucoepithelia	Sensory and cranial nerve ganglia
Varizella-zoster virus (VZV)	Human herpesvirus 3 (HHV-3)	Alpha, Varicellovirus	Mucoepithelia	Sensory and cranial nerve ganglia
Epstein-Barr virus (EBV)	Human herpesvirus 4 (HHV-4)	Gamma; Lymphocryptovirus	B and epithelial cells	Memory B cells
Human cytomegalovirus (HCMV; CMV)	Human herpesvirus 5 (HHV-5)	Beta; Cytomegalovirus	Epithelia, Endothelia, Monocytes and lymphocytes	Monocytes, Lymphocytes
Roseola virus (HHV-6A/-6B)	Human herpesvirus 6A and 6B (HHV-6/-6B)	Beta; Roseolovirus	T cells	Various leukocytes
Roseola virus (HHV-7)	Human herpesvirus 7 (HHV-7)	Beta; Roseolovirus	T cells	T cells, epithelia
Kaposi's sarcoma-associated herpesvirus (KSHV)	Human herpesvirus 8 (HHV-8)	Gamma; Rhadinovirus	Probably lymphocytes and epithelia	B cells

III.2 EBV and associated malignancies

EBV was the first human γ-herpesvirus discovered by Anthony M. Epstein, Bert G. Achong and Yvonne M. Barr (13). The virus was originally detected in cultured lymphoma cells from tumor samples, collected in Uganda by Dennis Burkitt, a British colonial surgeon. Epstein and Barr assumed a "passenger role" for the virus, since it persisted *in vitro* in dividing cells for many weeks (13) but it turned out that they found the first human virus with tumorigenic potential. This was soon confirmed by *in vitro* experiments, e.g. by the capability of EBV to transform B lymphocytes into lymphoblastoid cell lines (LCLs) with infinite proliferation capacity, supporting the potential role of EBV as an oncogenic virus (14). Based on specific criteria to establish a causal relation (15), such as I) elevated antibody titers to the virus prior to tumor development; II) presence of the viral genome within tumor but not in associated/adjacent normal cells; III) clonal nature of the viral genome; and IV) expression of viral genes in tumor cells, and due to its tissue tropism, it is not surprising that EBV was subsequently linked to various lymphoid and epithlelial cell malignancies (Table 2). Apart from Burkitt's lymphoma (BL) (16), EBV has been associated with Hodgkin's lymphoma

(HL) (17, 18), post-transplant lymphoproliferative disorder (PTLD) (19–22), nasopharyngeal carcinoma (NPC) (23–30) and a subset of gastric carcinomas (GC) (31–35) and several others as mentioned elswhere (5, 36, 37). EBV owes its oncogenic potential the latent infection cycle, whereas distinct latency programs with specific EBV gene expression patterns were characterized *in vivo* and *in vitro* (Table 2).

The oncogenic role of EBV in epithelial cell malignancies has been most extensively studied for NPC and GC. Aspects of EBV-associated NPC and GC and their oncogenesis are discussed and reviewed elsewhere (5, 24, 26, 31, 33, 38–40). The following sections will briefly summarize characteristics of EBV-associated NPC and GC with focus on common features and differences regarding oncogenesis and the causal role of EBV.

Table 2: EBV associated malignancies and characteristic latent gene expression patterns (adapted from (5)). AIDS = acquired immunodeficiency syndrome; UCNT = undifferentiated carcinomas of the nasopharyngeal type; VAHS = virus-associated hemophagocytic syndrome; EBERs = EBV-encoded RNAs; EBNA = Epstein-Barr nuclear antigen; LMP = latent membrane protein; a = intermediate latency type with variable levels of LMP expression (41); b = latency type I and 40% of the cases show LMP2 expression (42); c = latency pattern follows type II with LMP1 expression restricted to few cells (43).

Tumor	Subtype	Association with EBV [%]	Latent EBV genes expressed	Type of latency
Burkitt's Lymphoma	Endemic	100	EBERs, EBNA1	I
	Sporadic	>20		
	AIDS-associated	30-40		
Hodgkin's lymphoma	Mixed cell; lymphocyte depletd	60-80	EBERs, EBNA1, LMP1, LMP2	II
	Nodular sclerosing	20-40		
Post-transplant lymphoproliferative disease	Immunodefficient	100	EBERs, EBNA1, 2, 3A, 3B, 3C, LP, LMP1, LMP2	III
	Post-transplant	>90		
	AIDS-associated	>80		
Nasopharyngeal carcinoma	Undifferentiated, non-keratinizing	100	EBERs, EBNA1, (LMP1)[a], LMP2[a]	I/II [a]
	Undifferentiated, keratinizing	30-100		
Gastric carcinoma	UCNT	100	EBERs, EBNA1, (LMP2)[b]	I [b]; (II)
	Adenocarcinoma	5-15		
NK/T cell lymphoma	VAHS-associated	100	EBERs, EBNA1, (LMP1)[c], LMP2	II [c]; (I)
	Nasal	100		
Leiomyosarcoma	Immunodefficient	100	(EBERs, EBNA1, EBNA2, LMP1, LMP2) (44)	(Type III suggested) (44)
	Post-transplant	100		
	AIDS-associated	100		

III.2.1 EBV-associated NPC and GC

Pathology, incidence of EBV-associated NPC and GC

NPC usually presents as a neck mass or with symptoms of nasal obstructions and the loss of hearing and arises from the surface epithelium mainly of the lateral and superior walls of the nasopharynx. Due to its moderate early symptoms, NPC is rarely diagnosed before the age of 30 and male are disproportionately affected (2-3 male/female). According to the World Health Organization (WHO) NPC are classified into three types depending on their differentiation status and histology (45): keratinizing squamous cell carcinomas (WHO type I) that are highly differentiated with characteristic epithelial growth patterns and keratin expression; differentiated non-keratinizing carcinomas (WHO type II) with retained epithelial cell shape and growth patterns; and undifferentiated carcinomas (WHO type III) without keratin expression and indistinct growth pattern with intense infiltration of lymphoid cells referred to as *lymphoepithelioma* or *lymphoepithelial carcinoma*. Of all NPC cases about 20% are of the type I while the remaining 80% are type II and III NPCs. Type II and III NPCs are distinct from all other squamous cell carcinomas because of the universal association with EBV and are therefore also referred as EBV-associated NPCs. In most countries, NPC is a rare malignant disease with an age-adjusted incidence rate for both sexes of less than 1 per 100,000 (46). The disease is very common in southern China, especially among the Cantonese population of the Guangdong province, and Southeast Asia. In Hong Kong, an incidence rate of 20-30 per 100,000 and 15-20 per 100,000 among men and women, respectively, was reported for NPC (38) but with decreasing trend (~30%) (47). The incidence rate of NPC drops to 2-3 per 100,000 among men of the northernmost Chinese provinces (38). Additionally, an increased incidence of EBV-associated NPCs was identified among Arabs of North Africa and the Inuit population of Alaska and Greenland (38, 48). Interestingly, NPC is more frequently found among southern Chinese emigrants and less common among Chinese born in North America than those born in southern China (49, 50). This distinct geographical and ethnical distribution pattern strongly suggests an involvement of two etiological factors, genetic susceptibility and environmental factors, in the development of NPC in addition to EBV.

Similar to NPC, the majority of EBV-associated GCs (EBVaGCs) (~80%) are of the lymphoepithelioma type with a diffuse, poorly differentiated histology and dense infiltration of lymphoid cells. EBVaGCs are also described as undifferentiated carcinomas of the

nasopharyngeal type (UCNT). Macroscopically, EBVaGC often presents as an ulcerated or saucer-like tumor accompanied by marked thickening of the gastric wall (31, 51, 52). Thereby, the proximal stomach, the cardia and the middle stomach, are predisposed site for the development of EBVaGC (31, 39, 40, 53, 54). Whereas gastric cancer associated with *Helicobacter pylori*, causing chronic gastritis, intestinal metaplasia, and cancer, predominantly arises in the antrum part of the stomach. Thus, this pathogen might cause gastric cancer by independent mechanisms (31, 39). EBVaGC is, in contrast to BL and NPC that are endemic in equatorial Africa and southern China, respectively, a non-endemic malignant disease with a worldwide distribution. The incidence rates for EBVaGC differ regionally between 20.1% in Japan and 1.3% in Papua New Guinea (40). Recent meta-analysis showed that the prevalence of EBV was similar in cases from Asia (8.3%), Europe (9.2%), and America (9.9%), thus about 80,000 patients are estimated to develop EBVaGC (54). As with NPC, a predominance of EBVaGC in males was confirmed by meta-analysis (53, 54) and mostly younger individuals appear to be affected (53) although the age-association remains inconclusive (31, 39, 40).

Etiological factors apart from EBV

An association of the Human Leukocyte Antigen (HLA) alleles/haplotypes and the development NPC was consistently reported in many studies as reviewed elsewhere (24, 55). Especially HLA class I alleles, mostly of HLA-A and HLA-B subtypes specifically those prevalent within the Chinese but not in the Caucasian population, show a strong correlation with NPC and other EBV-associated malignancies (24, 55). Additionally, other genetic factor like chromosomal aberration and epigenetic changes seem to be crucial for NPC initiation and development as extensively reviewed elsewhere (26). Several studies showed numerical and structural abnormalities, e.g. deletions, duplications, translocations and rearrangements, leading to loss of heterozygosity (LOH) in the NPC genome (26). The most common genetic alterations in NPC are allelic deletions on chromosomes 3p and 9p (56–58). In NPC, LOH is often associated with inactivation of tumor suppressor genes like *p16* (*CDKN2A*), *Ras Association Domain Family 1A* (*RASSF1A*) and activation of oncogenes such as *Cyclin D1* (*CCND1*), *lymphotoxin-beta receptor* (*LTBR*), *phosphatidylinositol 3-kinase* (*PI3K*), *NOTCH3*, leading to deregulation of associated signaling pathways (26). The cell cycle regulators p16, inactivating cyclin D1/cdk4/pRb axis and inhibits cell cycle progression from G1- to S-phase, and cyclin D1, regulating G1- to S-phase transition, seem to play a pivotal role in NPC development. Activity of p16 is lost in more than 85% of NPCs, while CCND1 is

overexpressed in over 90% of the cases (26). Both features appear to be important for the establishment and the support of a stable EBV infection in nasopharyngeal epithelial cells (59–61). Epigenetic modifications, like promoter hypermethylation and histone acetylation, are additional mechanisms to regulate gene expression and are frequently detected in NPC (26).

EBVaGC show as well specific genetic and epigenetic alterations. However, data on chromosomal aberrations is rare and inconsistent. It was reported that gains in chromosome 11 and losses in 15q15 are more common in EBVaGC (62, 63), while zur Hausen *et al.* reported a significantly more frequent loss of chromosome 4p and of 11p that was exclusively restricted to EBVaGC (64). Epigenetic abnormalities, as already describes for NPC, are considered as key mechanisms promoting carcinogenesis of EBVaGC (33). Hypermethylation of CpG islands, leading to transcriptional silencing of tumor suppressor genes, are global and non-random in EBVaGC (33). In particular, a number of genes, such as *p16*, *p14* (*CDK2AP2*), *p73* (*TP73*), *E-cadherin* (*CDH1*), *RASSF1A* are frequently hypermethylated in EBVaGC (33, 40).

Besides the genetic factors, some environmental factors are correlated with NPC. The most prominent correlation is the association with the traditional diet of the southern Chinese population. Especially, Cantonese-style salted fish and other preserved foods contain volatile nitrosamines, serving as chemical carcinogens that may induce genetic damage in nasopharyngeal epithelial cells (24). The consumption of salted fish during childhood was shown to be related to an increased risk of NPC in southern China (38). The decreasing incidence rate in Hong Kong may be attributed to changes in life style, in particular the avoidance of salted fish in the diet of young children (24). However, there is only a weak or controversial association of NPC with other environmental factors like cigarette smoking and formaldehyde exposure (38).

Like with NPC several environmental factors are discussed in the context of EBVaGC. An interview study from Japan showed a significant correlation between frequent intake of salty food and an increased risk for EBVaGC (65). Other dietary factors like coffee drinking and high-temperature drinks as well as frequent exposure to wood dust and/or iron filings, which may induce mechanical injuries in the stomach membrane, correlated with an increased risk for EBVaGC, although not significantly (65).

III.2.2 EBV and the association with NPC and GC

The first link to NPC was established in 1974 by Klein and colleagues (66). They found EBV DNA in anaplastic cells of tumor biopsies from poorly differentiated nasopharyngeal carcinoma. These observations were confirmed by Anderson *et al.* in 1977 (67). Already in 1966, a correlation between EBV and NPC was suggested based on serological data, showing increased EBV antibody titer in sera of NPC patients (68). This was followed by the detection of EBV DNA in tumor biopsies from anaplastic carcinomas of the nasopharynx (69). Finally, the monoclonality of the EBV genome, based on the detection of terminal repetitive sequences by southern blot, was proven and the clonal expansion of a single EBV-infected progenitor cell was suggested as model for EBV associated epithelia cell malignancies (70). By contrast to NPC, the association of EBV with GC was recognized almost 20 years later. The first report about the detection of EBV DNA by polymerase chain reaction (PCR) in lymphoepithelioma-like carcinomas of the stomach was published in 1990 (71). In 1991, Shibata *et al.* (72) detected monoclonal EBV by PCR and *in situ* hybridization (ISH) of GC biopsies. Subsequently, EBV was correlated with an average of about 10% of all gastric cancers worldwide (39, 52, 53, 73, 74), while the frequency of EBV positive GC varied from country to country. This subset was then defined as EBVaGC. Interestingly, EBV alone does not transform epithelial cells *in vitro* and low-grade dysplastic precursor lesions in both NPC and EBVaGC have been shown to be EBV negative (32, 57, 58). This leads to more or less similar multistep models for the carcinogenesis of NPC and potentially as well for EBVaGC. These models suggest that preexisting cellular alterations generate cells that are either more susceptible to EBV infection or are able to support a persistent latent EBV infection, thus contributing to tumor progression and malignant transformation (24–27, 75) (Figure 1).

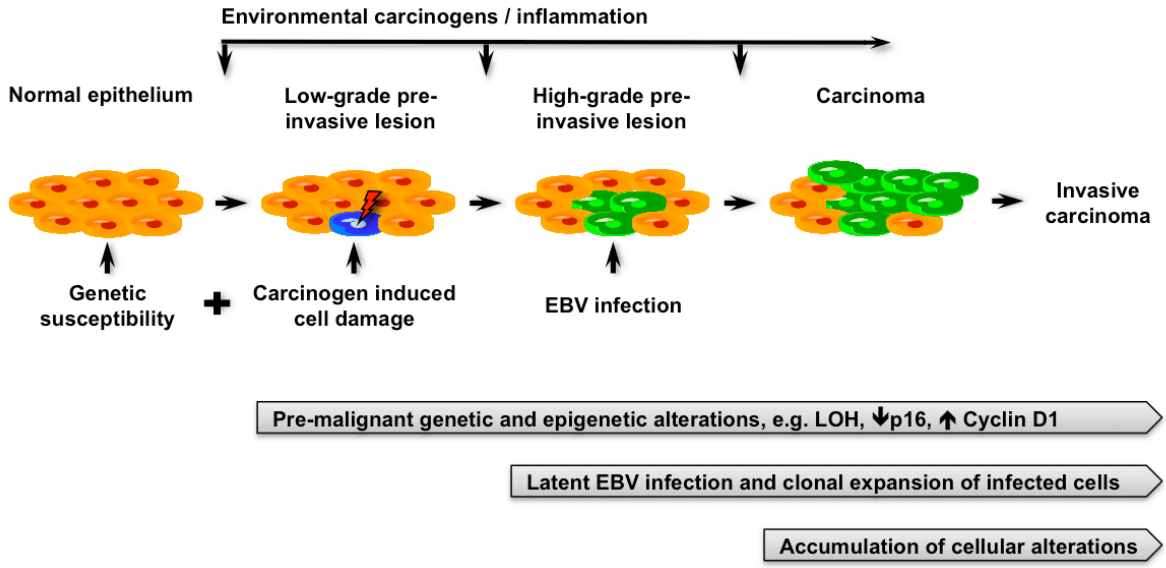

Figure 1: Multi-step tumorigenesis model for Epstein-Barr virus-associated epithelial malignancies such as nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). EBV infection alone cannot drive malignant transformation of normal, healthy epithelial cells *in vitro*. The healthy epithelium might harbor cells that are already susceptible to an infection by EBV or cells might become susceptible after induction of pre-malignant genetic and epigenetic alterations such as loss of heterozygosity (LOH), inactivation of tumor supressor genes and activation of oncogenes. These low-grade pre-invasive lesions, possibly a result of inherited genetic traits, environmental/dietary factors and inflammation, respectively, might support and facilitate the establishment of a latent EBV infection. The expression of latent EBV gene products leads to clonal expansion of infected cells and promotes malignant transformation. Upon carcinoma development, carcinoma cells might acquire further genetic and epigenetic alterations, leading to metastatic phenotype and therefore to invasive carcinoma.

III.2.3 EBV and its contribution to NPC and GC development

As mentioned above, EBV alone is not able to transform epithelial cells and the oncogenic potential of EBV is attributed to the latent EBV infection. Thus, EBV infection is considered as a late event in NPC and EBVaGC development (compare Figure 1). Latent EBV infection shows distinct gene expression patterns in NPC and EBVaGC (compare Table 2). NPC displays in general a type II latency pattern with expression of Epstein-Barr nuclear antigen 1 (EBNA1), the latent membrane proteins 1 and 2 (LMP1 and LMP2), EBV-encoded RNAs (EBERs) and several *Bam*HI A rightward transcripts (BARTs) including the lytic protein BARF1. However, the expression of the LMPs is variable (41). By contrast, EBVaGC shows an even more restricted expression pattern, reflecting latency type I with expression of EBNA1, EBERs and BARTs (including BARF1). Additionally, about 40% of all cases show expression of LMP2 (42). Key functions of the latency-associated EBV genes involved in NPC and EBVaGC are summarized in Table 3 and will be discussed in more detail within the following sections.

Table 3: Key functions of latency-associated EBV encoded RNAs and proteins involved in NPC and EBVaGC. EBERs = EBV encoded RNAs; BARTs = *Bam*HI A rightward transcripts, including the open reading frame (ORF) for BARF1 and additional putatively protein encoding ORFs; EBNA = Epstein-Barr nuclear antigen; LMP = latent membrane protein.

Latent EBV product	Key functions	Reviewed in
EBERs	Growth promotion; induction of type I interferons and pro-inflammatory cytokines	(76, 77)
BARTs	Maintenance of viral latency; resistance to apoptosis; immune evasion; oncogenesis	(76–80)
EBNA1	Maintenance of the viral episomal genome; growth promotion; metastasis promotion; immune evasion	(81)
LMP1	Growth promotion and cell cycle progression; resistance to apoptosis; promotion of invasion and metastasis; immune evasion; modulation of the tumor environment	(41, 75, 82)
LMP2	Maintenance of viral latency; suppression of cell differentiation; promotion of metastasis	(41, 83–85)

EBV-encoded RNAs (EBERs)

EBV encodes two small non-coding RNAs, EBER1 and EBER2, that are abundantly expressed (up to 10^7 molecules per cell) in each latently EBV-infected cell (1). Thus, EBER *in situ* hybridization (EBER-ISH) is a powerful tool to identify EBV-associated malignancies and is commonly used for diagnostics. EBERs were found to induce expression and secretion of insulin-like growth factor 1 (IGF-1) *in vitro* (86, 87). IGF-1 acts as autocrine growth signal and EBERs are therefore suggested to contribute cancer development and tumor growth *via* IGF-1 *in vivo*, since IGF-1 is constantly expressed in NPC biopsies and GC-derived cells express the IGF-1 receptor (76, 87). Additionally, EBERs can modulate the innate immunity. It was shown that EBERs can be recognized by the retinoic acid inducible gene I (RIG-I) and by TLR3, in combination with the EBER-binding protein LA (lupus antigen). Recognition of EBER leads to the induction of type I interferon (IFN), interleukin 10 (IL-10) and pro-inflammatory cytokines, respectively (88, 89). While IGF-1 and IL-10 can act as autocrine growth signals, the induction of type I IFNs and pro-inflammatory cytokine appears to be disadvantageous for EBV. However, EBERs confer resistance to IFN-induced, and Fas-mediated apoptosis by blocking protein kinase R (PKR) phosphorylation (90) and pro-inflammatory cytokine production might explain the high degree of lymphocyte infiltration in NPC and EBVaGC.

*Bam*HI A rightward transcripts (BARTs)

The *Bam*HI A region of the EBV genome contains a cluster of several transcripts called BARTs that are also abundantly expressed during EBV latency. BARTs are long,

multi-spliced transcripts that were originally discovered in cDNA libraries prepared from a serial passaged, xenografted NPC tumor (91). Differential splicing can produce several open reading frames (ORFs) putatively encoding for BARF0 and RK-BARF0, A73 and RPMS1. Although recombinant proteins with interesting functions could be generated from these ORFs *in vitro*, their biological relevance *in vivo* is questionable. None of them could be detected in tumors or EBV-infected cell lines (92, 93). However, recombinant BARF0 could be detected *in vitro* by immunoprecipitation using serum from NPC patients (94). An additional protein is encoded within the *Bam*HI A region, called BARF1. BARF1 is commonly found in NPC and EBVaGC (95, 96) but is considered to be a lytic protein since it is expressed upon induction of the lytic cycle within BL cells (97). However, BARF1 shows tumorigenic activity. Expression of BARF1 can induce malignant transformation in rodent fibroblasts, can immortalize primary monkey epithelial cells and enhances tumorigenicity of BL and NPC derived cell lines as summarized by Takada (76). In addition to BARF1 induced cell cycle activation, BARF1 induced activation of Bcl-2 contributes to its transforming capacity (76). While BARF1 is abundantly expressed in NPC and EBVaGC, EBV-encoded micro RNAs (miRNAs), so called miR-BARTS, appear more important for EBV-driven epithelial cell malignancies (31, 78–80, 98). MiR-BARTs are closely related to siRNA and can regulate gene expression either by blocking protein synthesis or by induction of mRNA degradation (99). Currently, 22 EBV-encoded miRNA precursors (miR-BART1-miR-BART22) are known to be expressed from two clusters within the BARTs and in principle can give rise to 44 mature miRNAs (78). MiR-BARTs are involved in regulation of both viral and host gene expression. The most important function of miR-BARTs is potentially maintenance of EBV latency, since EBV is believed to undergo lytic replication within oropharyngeal epithelial cells, which is also the reason why they are believed to be the source of infectious progeny virus (5, 100, 101). The miR-BART2-5p was shown to target BALF5 (102), the viral DNA polymerase that is responsible for EBV-DNA replication during the lytic replication cycle, leading to suppression of the lytic cycle and EBV maintenance. Other viral targets of miR-BARTS are LMP1 (103–105), the major EBV oncogene, and LMP2A (106), the most immunogenic viral protein in latency I and II infections and involved in cellular transformation. MiR-BART-mediated fine-tuning of LMP1 expression balances its pro-apoptotic and oncogenic effects and increases cellular proliferation, thus promoting cancer development. In contrast, down-regulation of LMP2A expression increases immune evasion. Additionally, most of the EBV-encoded miRNAs target host cell genes involved in apoptosis, immune recognition and tumor suppression as summarized by Lung et al. (79). Taken

together, miR-BARTs are important for EBV maintenance, immune evasion, prevention of apoptosis and oncogenesis in EBV-associated epithelial cell malignancies (79).

Epstein-Barr Nuclear Antigen 1 (EBNA1)

EBNA1 plays multiple important roles and is expressed in all proliferating latently EBV-infected cells (81). The main function of EBNA1 is maintenance of the EBV episome and its mitotic segregation to the daughter cells (107, 108). EBNA1 binds as dimer to specific elements, family of repeats (FR) and dyad symmetry (DS) elements, within the EBV *oriP* and ensures episomal replication once per cell cycle (109, 110). Additionally, EBNA1 can act as transcriptional activator for the LMP genes (111). Expression of EBNA1 alone was shown to increase primary tumor and metastasis formation by NPC cells and enhanced tumorigenicity of GC cells (112, 113). Several signaling pathways, known to regulate cell growth and transformation, are as well affected by EBNA1 (81). For example, EBNA1 was shown to disrupt Promyelocytic leukemia (PML) nuclear bodies and modulate p53 levels in NPC and GC cells (114, 115), leading to resistance against p53-mediated and DNA-damage induced apoptosis, thus promoting cell survival. Interestingly, EBNA1 contains a glycine-alanine repeat sequence that serves as an inhibitor of HLA-I restricted antigen presentation and processing, hence leading to immune evasion (116).

Latent Membrane Protein 1 (LMP1)

LMP1 is considered the major oncogene of EBV since it has strong transforming properties in cultured cell lines and is one of five key viral proteins for B-cell transformation (41, 84). However, LMP1 is not expressed in EBVaGC while LMP1 transcripts are found in a majority of NPCs, although protein expression is detected only in 20-60% of the NPC tumors and amongst those sometimes only a small subset of cells show LMP1 protein expression (34, 117, 118). Nevertheless, LMP1 can engage multiple signaling pathways contributing to cell proliferation, survival, motility and invasion. The function of LMP1 is strongly dependent its expression levels (75, 119, 120). The oncogenic properties of LMP1 are thereby mostly attributed to its function as constitutively active tumor necrosis factor (TNFR), by mimicking TNFR1 and CD40, in a ligand independent manner (41, 75, 82). LMP1 is an integral transmembrane protein and self-aggregation or oligomerization, which is driven by the hydrophobic transmembrane (TM) domains, is essential to activate intracellular signaling pathways (75, 82). TM domain mediated recruitment of the Rho-GTPase Cdc42 leads to

cytoskeletal remodeling and induction of signaling pathways involved in cell proliferation and autophagy (121). The most profound effects of LMP1 are mediated by the C-terminal cytosolic tail, which contains 3 functional domains termed C-terminal activating regions 1-3 (CTAR1-3). CTAR1-3 possess specific docking sites for signaling adaptor proteins, including the tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), TNFR-associated death domain (TRADD), receptor interacting protein kinase (RIP), BS69 and Janus kinase (JAK)-3 proteins (41, 82, 85). This results in signal transduction through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), through c-Jun N-terminal kinase (JNK)/p38-Stress-activated protein kinases (SAPK), through Phosphatidylinositol 3 kinases (PI3K)/Akt, through extracellular-signal-regulated kinase **(**ERK)-Mitogen-activated protein kinase (MAPK) and through Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathways and through induction of specific genes that are involved with apoptosis, cell cycle progression, cell proliferation, immortalization, invasion, migration and modulation of the tumor environment (41, 82, 85). An important additional target of LMP1 is the epidermal growth factor receptor (EGFR) (122, 123). Thus, LMP1 is involved in a wealth of cellular processes, as reviewed in great detail elsewhere (41, 75, 82) and might be crucial at least in the early stages of NPC development (28) by contributing to tumor progression. Interestingly, LMP1 was found to induce telomerase activity (82, 124). Telomerase expression and activity is in general a hallmark of cancer (125, 126) and interestingly, basal epithelial stem and progenitor cells within the basal layers of epithelial tissues show increased telomerase activity (127–132) as discussed later.

Latent Membrane Protein 2 (LMP2)

The EBV gene *LMP2* encodes for two distinct proteins, LMP2A and LMP2B. Both proteins are integral transmembrane proteins like LMP1. Although both proteins share common or overlapping features, LMP2A possesses almost exclusively signaling capabilities, while LMP2B was suggested to negatively regulate LMP2A-mediated signaling (133). It is now widely accepted that LMP2A regulates and promotes EBV latency by blocking B cell receptor (BCR) mediated signaling and lytic reactivation of EBV (134, 135). Additionally, LMP2A promotes proliferation and survival in B cells (136). Responsible for LMP2A mediated signaling are specific motifs at the N-terminal cytosolic tail (41). These motives contain numerous tyrosine residues that constitute functional immunoreceptor tyrosine-based activation motifs (ITAM) and proline/tyrosine (PY)-motifs, capable of recruiting several signaling molecules like the tyrosine kinase Lyn, Syk, and the ubiquitin ligases Nedd4/Itchy.

These molecules lead to the activation of several signaling pathways, e.g. the PI3K/Akt, the JNK/SAPK, the ERK-MAPK and the Wnt/β-catenin, which promote cell growth, inhibit apoptosis and differentiation, and contribute to cell transformation (41). Especially LMP2A-activated PI3K/Akt signaling appears to be important in epithelial cells regarding tumor development (41, 85). It was shown that LMP2A can block cell differentiation, enhance cell adhesion and cell motility, induce transformation and anchorage-independent growth through PI3K/Akt activation (137–142). Notably in EBVaGC, LMP2A can induce STAT3-mediated up-regulation of DNA methyltransferase 1 (DNMT1), leading to promoter hypermethylation and silencing of tumor suppressor genes (83).

Exosomes

Interestingly, EBV can modulate the tumor environment by induced secretion of exosomes. Exosomes are small vesicles of unique size and shape, known to contain transfer and messenger RNAs as well as proteins and other molecules. Exosomes released from EBV-infected cells can contain LMP1, EBERs and BARTs, thus impacting on surrounding cells by their described functions (75, 76, 78, 85) and contributing to immune evasion (11, 80).

III.3 EBV infection mechanisms of B cells and epithelial cells

Before EBV can develop its full oncogenic potential, it first has to infect an epithelial cell to drive malignant transformation, thus contributing to development and progression of tumors such as NPC and EBVaGC. While the steps involved in the infection of B cells are quite well studied and understood (143, 144), the attachment and entry mechanisms of EBV into epithelial cells still remain to be fully elucidated.

III.3.1 Infection of B cells by EBV

The infection of B cells by EBV is a multistep process (Figure 2), requiring endocytosis (143, 144). The first step is attachment of EBV to B cells mediated by the cellular complement receptor type 2 (CR2; also known as cluster of differentiation 21 or CD21) and the viral glycoprotein complex gp350/220 (encoded by *BLLF1*). Subsequently, the viral glycoprotein gp42 (encoded by *BZLF2*) binds to HLA class II molecules, which serve as co-receptor on the B cell. Gp42 is noncovalently associated with the viral glycoprotein complex gHgL (also known as gp85/gp25 encoded by *BXLF2*/*BKRF2*) that is part of the viral core fusion machinery in addition to the glycoprotein gB (also known as gp110 encoded by

BALF4). Binding of gp42 to HLA-II molecules triggers fusion of the viral and the endosomal membrane and finally allows entry of the tegumented EBV capsid into the cytoplasm of the B cell (143, 144). Interestingly, an additional attachment receptor for EBV, CD35 (or CR1), was recently identified in a patient with genetic CR2 deficiency (145) and thus potentially leading to a broader spectrum of EBV-susceptible cells.

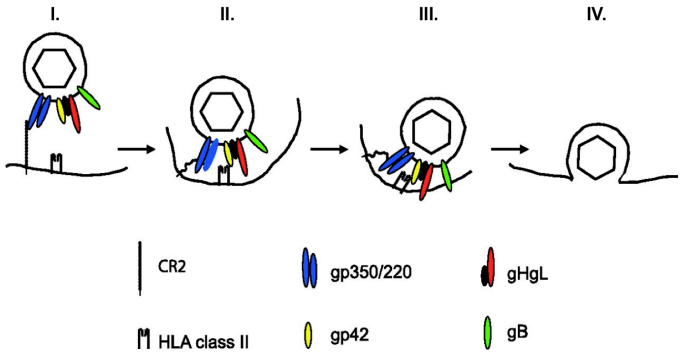

Figure 2: Multi-step model for the infection of B cells by EBV (from reference (143)**; adapted).** I.) Binding of EBV *via* gp350/220 to CR2 on the B cell surface and triggering of endocytosis. II.) The potential flexibility of CR2 may allow the EBV to approach closer to the cell membrane, where gp42 can interact with HLA class II molecules. III.) Interaction of gp42 with HLA class II subsequently triggers the interaction of the core fusion machinery, gHgL and gB, with the endosomal membrane. IV.) Fusion of viral and endosomal membranes, which allows entry of the tegumented capsid into the cytoplasm.

III.3.2 Infection of epithelial cells by EBV

In contrast, the infection of epithelial cells by EBV is more complicated. Epithelial cells might express low levels of CR2 *in vitro* (146) but which, or even whether, epithelial cells normally express CR2 *in vivo* remains uncertain. Thus EBV has to employ a different strategy to infect an epithelial cell (Figure 3). Currently, there are several different mechanisms for the infection of epithelial cells by EBV proposed (143). The fist CR2-independent EBV entry mechanism into epithelial cells was demonstrated in 1992 by Sixbey *et al.* (147). EBV-specific polymeric immune globulin A (IgA), commonly present in saliva, could mediate endocytosis-transfer of EBV into epithelial cells by binding of the J (joining) chain to the secretory component (SC) protein. However, this mechanism might be particularly relevant for EBV entry from the basolateral site or for transcytosis of EBV through epithelial cells since the SC protein is usually present at the basolateral surface of epithelial cells (147, 148). As a second mechanism it was demonstrated that B cell-derived EBV can bind to CD21-negative epithelial cells directly *via* the gHgL complex of the core fusion machinery (149, 150). Although the underlying mechanism and the receptor on the epithelial cell were not determined and identified, respectively, these observations indicated the presence of an epithelial receptor for gHgL (referred as gHgLR; potentially integrin αVβ5, αVβ6 and αVβ8 as described below). The third, and probably the most intriguing mechanism,

involves integrins expressed on epithelial cells. Integrins are well-know virus attachment factors (151, 152). Tugizov and colleagues showed that the viral transmembrane protein BMRF2 can interact with epithelial cell integrins (153), mediating infection of these polarized oropharyngeal epithelial cells from the basolateral surface. Subsequent studies confirmed that the RGD-motif, present at the exposed loop of BMRF2, is a ligand for β1, α3, α5, and αV integrins and that BMRF2 can facilitate cell-to-cell spreading of EBV (154–156). Additionally, integrins appear to be involved in fusion of EBV with epithelial cells (144, 157). It was shown that binding *via* the KGD-motif of gHgL to αVβ5, αVβ6 and αVβ8 can trigger membrane fusion in cell-based assays and that binding induces conformational changes of these integrins (158, 159). Interestingly, it was recently demonstrated that EBV could be transcytosed through polarized epithelial cells in both directions, from the apical to the basolateral surface and *vice versa* (160). This implicates another important alternative mechanism involved in the life cycle of EBV, independent of productive infection in epithelial cells.

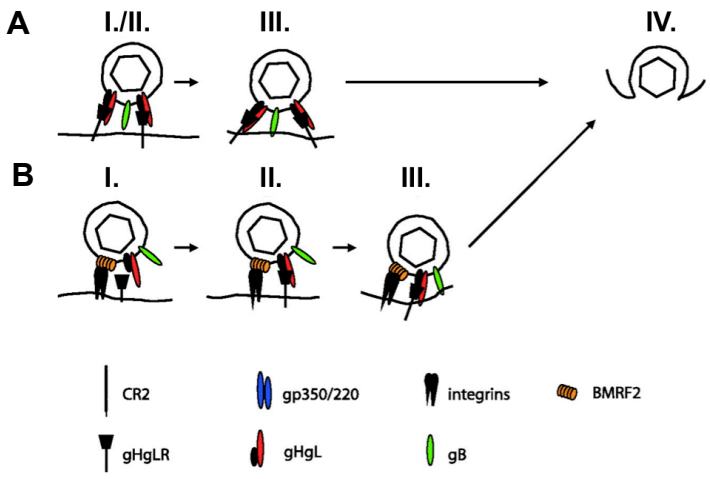

Figure 3: Models for CR2-independent infections of epithelial cells by EBV (from reference (143)**; adapted). A:** Entry *via* gHgL alone (less efficient). I./II.) EBV binds directly *via* gHgL to gHgLR (potentially integrins αVβ5, αVβ6, and αVβ8). (3) Binding triggers the interaction of the core fusion machinery, gHgL and gB, with the cell membrane. **B:** Attachment and entry *via* BMRF2 and gHgL. I.) BMRF2 interacts with α5β1 integrins. II.) gHgL interacts with gHgLR. III.) Binding triggers the interaction of the core fusion machinery with the cell membrane. Final step is the same for both mechanisms. IV.) Virus and cell membrane fusion leads to release of the tegumented EBV capsid into the cytoplasm.

III.3.3 EBV infection and the interplay between epithelial cells and B cells

Thus, EBV obviously employs different mechanisms to infect B cells and epithelial cells. The viral glycoprotein gp42 is necessarily involved in B cell infection. By contrast, gp42 is not essential for epithelial cell infection. Moreover, gp42 impedes infection of epithelial cells (161). Interestingly, EBV particles released from B lymphocytes show low levels of gp42 and are more infectious for epithelial cells, while virions made in epithelial cells possess high levels of gp42 and are better able to infect a B cells. This led to a concept

where EBV shuttles between B cells and epithelial cells during its life cycle, thus undergoing a switch in cell tropism (143). This concept supports the role of pharyngeal epithelial cells in lytic EBV replication and release of infectious virus for transmission. Thus implicating that transmitted EBV virions, shed by epithelial cells, would need to infect B cells directly within the oro- and nasopharyngeal mucosa of the next susceptible host. For example, this could occur within the nasopharyngeal-associated lymphoid tissue (NALT), which is well known to be heavily infiltrated with lymphocytes. However, the infection of epithelial cells *in vitro* is difficult to achieve using cell free EBV. It was suggested that successful and efficient infections of epithelial cells could be achieved by using cell-to-cell contact and B-cell mediated transfer infection approaches (162, 163). Although the infection frequencies could be increased 10^3 to 10^4 fold compared to infections using cell free virus, this was not or only in part confirmed in other studies on primary epithelial cells (164, 165). The underlying mechanism still needs to be elucidated but the hypothesis is that binding of EBV to B cells, *via* the interaction of gp350 and CD21, induces conformational changes within the viral envelope, therefore leading to exposure of viral ligands, e.g. BMRF2 or gHgL, to an putative epithelial receptor such as integrins (25, 143, 163). This is supported by the finding that antibodies against the viral gp350/220 can actually facilitate the infection of epithelial cells (147, 148, 166). A subsequent study revealed that the so-called B cell-mediated transfer infection is restricted to the basolateral surface of polarized epithelial cells (167). However, this finding appears not to be in line with the previous results of Tugizov *et al.* (153), who showed efficient cell-to-cell contact infection at the apical surface of polarized epithelial cells, although they used infected lymphocytes and not virus-coated B cells.

Taken together, the biology of EBV infection in epithelial cells, including the precise infection mechanisms of epithelial by EBV and the role of the epithelium within the life cycle of EBV remains puzzling. The significance of the epithelium in the life cycle of EBV will be addressed within the discussion section of this thesis and a putative role as transit route for the epithelium within the life cycle of EBV will be suggested.

III.4 Epithelial barrier as portal of entry for microorganisms and EBV

The fundamental role of any epithelia is to provide barriers between different compartments of the organism and to the outside environment. The fact that over 90% of infections occur at or through mucosal surfaces highlights the significance of epithelia as fist-line of defense against invading pathogens (168). Especially the epithelia lining the

pharyngeal cavities play an important role as physical and mechanical barriers against various environmental stimuli and invading microorganisms such as bacteria, fungi and viruses. Since EBV is transmitted *via* saliva and enters the host through the oral route, the epithelia within the pharyngeal cavities are as well the portal of entry and exit for EBV. The nasopharyngeal and palatine tonsils can be considered as gatekeepers and scavengers within the pharyngeal cavities and are part of the NALT within the Waldeyer's ring. They might represent the perfect target organs for EBV entry into the body since they are extensively infiltrated with lymphocytes (169, 170). In addition, NALT plays a crucial role in host defense against invading pathogens in the upper respiratory tract by serving as an interface between innate and adaptive immune responses (170).

III.4.1 Epithelial tissue architecture within the pharyngeal cavities

There are distinct epithelia within the pharyngeal cavities. Whereas some mucosal regions are covered by a keratinized epithelium resembling epidermis, other regions are lined by a non-keratinizing epithelium (171). Regions that are subjected to mechanical forces associated with mastication such as the gingiva and hard palate, posses a keratinizing epithelium. The remaining soft tissues of the pharyngeal cavity are covered with non-keratinized, stratified squamous epithelia. The stratified squamous epithelium is a dynamic tissue of distinct multilayer architecture (132, 171) with various patterns of differentiation (or maturation) between the deepest cell layer (undifferentiated) and the surface (differentiated). Undifferentiated epithelial cells within the basal layer of the epithelium, the *stratum basale*, are attached to the basement membrane by integrin-containing focal adhesions and are most important for tissue homeostasis. The basement membrane separates the epithelium from the underlying *lamina propria* and ensures correct and directed migration and differentiation of the overlying epithelial cells towards the surface of the epithelium. Differentiation involves migration accompanied by a loss of integrin expression and an increase in cadherin-mediated adhesion *via* close intercellular tight junctions and desmosomes (171). These tight intracellular connections separate the apical from the basolateral cell surface regions and ensure establishment and maintenance of cell polarity (170), thus contributing to tissue integrity and barrier function. The *lamina propria* contains blood vessels, minor salivary glands, structural fibers, nerves, fibroblasts and other cells like lymphocytes and plasma cells (172). As mentioned already, the *stratum basale* harbors a small sub-population of epithelial stem cells, which can undergo mitotic division and give rise to transiently proliferating progenitor cells (132, 173). These cells then can generate daughter cells that migrate and

differentiate through the *stratum spinosum* and *stratum granulosum* towards the epithelial surface, the *stratum corneum*.

III.4.2 Telomerase and the epithelium

Dividing cells face two problems: I) the chromosome end-protection and II) the chromosome end-replication. The ends of linear chromosomes, such as those in eukaryotes, must be distinguished from broken DNA ends that require repair. Additionally, the gradual loss of sequence information at the terminal end of chromosomes, owing to incomplete replication by DNA polymerases, must be prevented (174). These problems are solved by the ribonucleoprotein telomerase and associated telomeric proteins. Telomerase consists of the catalytically active telomerase reverse transcriptase (TERT) and the template-containing RNA component (TR or TERC). The main telomerase function is to ensure chromosomal maintenance in dividing cells by synthesizing and adding multiple tandem repeats of DNA, so called telomeric DNA, to the chromosomal ends. Additionally, recruited telomeric proteins further protect the linear chromosomal ends (174). Despite its main function in chromosomal maintenance, growing evidence suggest several additional, telomere-independent activities of TERT (175–177). TERT can act independently from TERC and its enzymatic activity and is involved in many biological processes, e.g. regulation of gene expression, promotion of cell proliferation, modulation of DNA-damage response, inhibition of apoptosis and inflammation, thus contributing to cancer development. For example, TERT can directly modulate NF-κB and Wnt-dependent gene transcription (178–180). Nevertheless, telomerase expression and activity ensure indefinite proliferation and self-renewal capacity and is usually restricted to stem and progenitor cells and is considered to be a hallmark of cancer in general (125, 126).

As mentioned above, a small sub-population of epithelial cells residing on the basement membrane of epithelial tissues possesses stem cell characteristics, like indefinite proliferation and self-renewal capacity. These epithelial stem cells give rise to transiently proliferating cells that either reside within the stem cell compartment or migrate and differentiate towards the epithelial surface (173) and are therefore crucial for epithelial tissue homeostasis. The self-renewal capacity of these indefinitely proliferating cells is ensured by increased telomerase expression and activity, which is lost while the cells migrate and differentiate (127–132). Additionally, due to their self-renewal and proliferation capacity, the epithelial stem and progenitor cells are more likely to aquire genetic and epitgenetic

alterations during mitotic divisions (132), thus generating an environment facilitating an establishment of an EBV infection as disscussed above and in manuscript I.

III.4.3 Telomerase and EBV

To gain access to its preferred target cell for a persistent latent infection, the B cell, EBV has to overcome the epithelium. Therefore it is likely that EBV encounters epithelial cells with increased telomerase expression and activity. EBV was shown to preferentially infect differentiated epithelial cells obtained from the sphenoidal sinus (sECs), to replicate lytically in differentiated oropharyngeal and tonge epithelial cells and as well as in tonsillar plasma cells (164, 181–184), indicating a dependence of the EBV infection on the differentiation status of the cells. By contrast, latent EBV infection in epithelial cells is restricted to and associated with EBV-associated epithelial cell malignancies as discussed above. Recently, it was shown that telomerase-immortalized nasopharyngeal epithelial (NPE) cell clones are able to support a long-term infection by EBV (60, 185). Ectopic expression of human TERT (hTERT) in combination with LMP1 extended the life span of primary nasopharyngeal epithelial cells and contributed to their immortalization (185). Loss of p16 activity and overexpression of CCD1, which putatively occurs prior to the infection by EBV, thereby appears to be crucial for the establishment and the support of a stable EBV infection (25) as discussed above. More recently, it was shown by Wille *et al.* that undifferentiated telomerase-immortalized normal oral keratinocytes (NOKs) supported a long-term latent EBV infection (186). Additionally, so far unpublished data from the same group apparently shows that a lytic EBV infection is preferentially found in more differentiated cell layers while undifferentiated basal layers of NOKs remain latently infected as mentioned elsewhere (7).

However, it is not clear in which way an increased telomerase activity in epithelial cells might actually be beneficial for EBV. It is possible that epithelial cells with increased telomerase activity are more susceptible to an infection by EBV or that an increased telomerase activity might be required for EBV to establish an infection in epithelial cells. Moreover, it was shown that expression of hTERT inhibits lytic EBV replication and that hTERT silencing can trigger lytic EBV replication in B cells (124, 187, 188).

III.4.4 Innate immunity and the epithelium

The innate immune system represents the first line of defense against invading pathogens apart from the mechanical and physical barrier function of the epithelium. The

innate immunity is crucial for mounting a rapid response against various kinds of microorganisms that are potentially harmful and is responsible to prime and shape the subsequent adaptive immunity. Amongst others, the innate immune system comprises anti-microbial effector molecules such as proteins, peptides, cytokines, chemokines and interferons as well as cellular receptor molecules and specific cell types like macrophages, dendritic cells, natural killer (NK) cells and others (189–193). As mentioned before, epithelial cells play a pivotal role for the innate immune system due to their strategic location at the border between the exterior environment and the interior of the body. Epithelial cells of the nasal and pharyngeal cavities can sense invading pathogens especially *via* a broad range of cell-surface and intracellular pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) (189, 191, 193). These PRRs recognize common, so called pathogen-associated molecular patterns (PAMPs) and binding of such PAMPs by PRRs triggers signaling pathways, leading to the expression of anti-microbial effecter molecules like pro-inflammatory cytokines, chemokines and interferons or triggers endocytosis as it is the case for CLRs. Nevertheless amongst these, TLRs were the first PRRs to be identified. They have been named after the Toll protein of the fruit fly (*Drosophila melanogaster*), which was originally described as important factor in embryonal dorso-ventral patterning (194) and subsequently found to be essential in the protection of fungal infections (195), indicating its contribution to innate immunity. Soon thereafter, the first human homologue was identified (196). Until today 10 human TLRs have been identified including their specific PAMPS or ligands except for TLR10 as summarized in Table 4.

In summary, TLRs recognize PAMPS from a wide range of microbes such as viruses, bacteria, mycobacteria, fungi and parasites and can be divided into two groups according to their location within distinct cellular compartments. The first group, consisting of TLR1, 2, 4, 5 and 6 is expressed in the plasma membrane at the cell surface and is involved in recognition of proteins, lipopeptides and polysaccharides. The second group comprises the nucleic acid sensors TLR3, 7, 8 and 9 that are exclusively located in intracellular vesicles such as the endosomes, endoplasmatic reticulum, lysosomes and endolysosomes (197).

Table 4: Toll-like receptors (TLRs) and their corresponding recognized pathogen-associated molecular patterns (PAMPs) and cellular location (adapted from (197, 198)**).** * = heterodimers.

TLR	Location	PAMPs/ligands (source)
1/2*	Plasma membrane (cell surface)	Triacyl lipopeptides (bacteria and mycobacteria)
2 (1 or 6)*	Plasma membrane (cell surface)	Peptidoglycan (gram-positive bacteria); Lipoarabinomannan (mycobacteria); structural proteins (viruses); phospholipomannan (fungi); Glycosylphosphophatidyl inositol mucin (Trypanosoma)
3	Endosome	Single-stranded RNA and double-stranded RNA (viruses)
4	Plasma membrane (cell surface)	Lipopolysaccharides (gram-negative bacteria); Mannan glycoinositolphospholipids (Trypanosoma), structural proteins (viruses)
5	Plasma membrane (cell surface)	Flagellin (flagellated bacteria)
6/2 *	Plasma membrane (cell surface)	Diacyl lipopeptides (Mycoplasma), LTA (Streptococcus), Zymosan (fungi); β-glucan (fungi)
7	Endosome	Single-stranded RNA (viruses)
8	Endosome	Single-stranded RNA (viruses)
9	Endosome	Double-stranded DNA viruses; unmethylated CpG motifs (bacteria and viruses); Hemozoin (Plasmodium)
10	Probably plasma membrane (cell surface) (199, 200)	Unknown

TLRs are type I transmembrane proteins that form homo- or heterodimers, as it is the case for TLR1, 2 and 6. They consist of an ectodomain that contains leucine-rich repeats and mediate the recognition of PAMPs, a transmembrane domain and cytosolic Toll-Interleukin 1 (IL-1) receptor (TIR) domains that activate downstream signaling pathways by binding of distinct adaptor molecules for each TLR. TLR signaling finally leads to the activation of transcription factors such as NF-κB and interferon regulatory factors (IRFs) that induce expression of anti-microbial effector molecules like pro-inflammatory cytokines, chemokines and type I interferons such as IL-6, TNF-α and IFN-α/β (198).

III.4.5 Toll-like receptors, EBV recognition and impact on EBV infection

Herpesviruses are sensed by different TLRs and can interact with TLRs and TLR signaling, respectively, by blocking TLR signaling and corresponding immune responses, thus leading to immune evasion (201, 202). Until now, EBV itself has been shown to activate and modulate TLR2, 3, 7, and 9 signaling in various cell types (89, 202–207). However, there is evidence that TLR signaling can impact on EBV infection itself. Following EBV infection, it was demonstrated that activation of TLR9 *via* exogenous stimulation contributes to efficient transformation of B cells (208). Furthermore, activation of TLR9 and TLR7/8 signaling increased EBV-driven B cell proliferation and transformation (209, 210). Whereas TLR3 stimulation in combination with an inhibiting drug against IAP (inhibitors of apoptosis)

elicited strong cytotoxic effects and inhibited proliferation of various EBV-positive and EBV-negative NPC cell lines and xenografts (207). In addition, our group recently demonstrated that immune activation *via* TLR9 triggering could inhibit the switch from latent to lytic EBV upon de novo *in vitro* infection of cord blood B cells (211) and upon B cell receptor (BCR)-induced reactivation of EBV in chronically infected BL cell lines (212). More recently, we demonstrated that in contrast to TLR9 activation, triggering of TLR2 with heat-killed group A streptococci (GAS) can induce lytic EBV reactivation in latently infected lymphoblastoid cell lines (LCLs), established from tonsillar mononuclear cells (TMCs) (213). Moreover, a very recent study shows that activation of TLR2, 3 and 4 in BL cell lines lead to up-regulation of lytic EBV genes (214). These results implicate that EBV could utilize TLR signalling potentially for I) driving latency, thus contributing to immune evasion and oncogenisis and for II) switching to lytic infection to ensure generation and amplification of progeny virus particle for transmission. Notwithstanding, these effects seem to be dependent on various factors, e.g. the physiological site of infection, the employed TLR and potentially the infected cell type. Although it is known that epithelial cells, including those of tonsils and upper airways, express TLRs and are capable of mounting innate immune responses (128, 191, 193, 207, 215–222), the impact of TLR signaling on EBV infection in epithelial cells remains elusive.

IV SUBJECT OF INVESTIGATION

EBV is a γ-herpesvirus with marked tropism for B cells. However, epithelial cells play an important role in the life cycle of EBV. Therefore, following aspects of the EBV infection in epithelial cells were subject of investigation within this thesis:

1. What is the impact of hTERT expression and telomerase activity on EBV infection of epithelial cells?

Increased telomerase activity is characteristic for epithelial stem and progenitor cells within the basal layers of epithelial tissues and a hallmark of cancer in general. Since EBV is transmitted *via* saliva, it has to overcome the epithelial barrier of the pharyngeal cavities, the portal of entry and exit for EBV, either to get access to the preferred B cell or to be spread to the next susceptible host. Additionally, EBV shows a tight association with epithelial cell malignancies such as nasopharyngeal and gastric carcinoma. However, EBV alone does not transform epithelial cells efficiently and is frequently lost from epithelial cells *in vitro*. We therefore investigated the impact and the contribution of hTERT expression and telomerase activity on the EBV infection in epithelial cells.

2. What is the influence of hTERT expression and differentiation status on the susceptibility of epithelial cells to an infection by EBV?

The infection by EBV relies mostly on attachment to the cellular receptor CD21 that is present on B cells but is only weakly or usually not at all expressed in epithelial cells. Additionally, integrins are well-known attachment receptors for various viruses and an involvement of certain integrins was demonstrated in the EBV infection of epithelial cells. Thus, we determined the integrin expression patterns in various epithelial cell lines and investigated whether increased hTERT expression or the differentiation status of primary epithelial cells from the sphenoidal sinus has an impact on expression of integrins that are involved in the infection of epithelial cells by EBV.

3. What is the influence of TLR9 activation on EBV infection in epithelial cells?

Various pathogens constantly challenge the pharyngeal cavity, the portal of entry for EBV. As part of the innate immune system, Toll-like receptors (TLRs) can sense specifically pathogen-associated molecular patterns and are therefore the first line of defense against

invading microorganisms. Epithelial cells within the pharyngeal cavity are known to express TLRs and to be capable of mounting innate immune responses. Recent studies of our group showed that activation of TLR9 can inhibit lytic reactivation in latently infected Burkitt's lymphoma cells of EBV while TLR2 ligation in EBV-transformed lymphoblastoid cell lines can trigger lytic EBV replication. This prompted us to investigate the impact of TLR9 activation on EBV infection in epithelial cells.

V RESULTS

V.1 Manuscript I: Telomerase activity enhances Epstein-Barr virus gene expression and contributes to virus maintenance in epithelial cells

Jürgen Rac, Florian Haas, Andrina Schumacher, Michele Bernasconi, Roberto F. Speck, David Nadal

Manuscript in preparation

V.1.1 Abstract

Epstein-Barr virus (EBV) is a ubiquitous virus, tightly associated with various lymphoid and epithelial malignancies. Epithelial stem cells and transiently proliferating cells within the basal layers of epithelial tissues exhibit unlimited self-renewal capacity mediated by activated telomerase activity. During cell division, these cells might acquire genetic and epigenetic alterations generating an environment to support persistent EBV infection. Amongst these alterations, loss of p16 and overexpression of cyclin D1 appear to be crucial for the establishment of an EBV infection in epithelial cells and are known to be common features in EBV associated epithelial tumors. However, the impact of telomerase activity on the infection of epithelial cells remains to be elucidated. Therefore, we generated epithelial model cell lines with increased telomerase activity by stable ectopic expression of hTERT, the rate-limiting component of the telomerase complex. Conversely, we suppressed telomerase activity by stable expression of a catalytically inactive, dominant negative hTERT mutant. We then performed infection experiments using a recombinant wild type EBV strain encoding GFP and determined infection rates by flow cytometry as well as EBV gene expression by RT-qPCR. We found that the infection is strongly depending on the cellular context and that infection frequencies partly depend on telomerase activity. Moreover, epithelial cells with increased telomerase activity showed up-regulation mainly of latent EBV genes as compared to their corresponding control cell lines. We conclude that increased telomerase activity directly acts on the EBV infection of epithelial cells by facilitating latent EBV gene expression and therefore contributing to EBV maintenance in epithelial cells.

For detailed information see attached Manuscript I.

V.1.2 Complementary Data and Results to Manuscript I

Integrin expression patterns in various epithelial cell lines

Epithelial cells commonly lack or show only low expression of CD21 (146), the main receptor for EBV attachment. Adhesion molecules such as integrins are well known attachment receptors for various viruses (151, 152). Several studies showed involvement of αV, α3, α5, β1, β6 and β8 integrins in the infection of epithelial cells by EBV (153–155, 159). The RGD motif of the viral transmembrane envelope glycoprotein BMRF2 was shown to bind to integrin αV, α3, α5 and β1 of oral epithelial cells (153, 155) and the integrins αVβ6 and αVβ8 appear to trigger membrane fusion via the KGD motif of the transmembrane envelope glycoprotein complex gH/gL of EBV (159). To identify and characterize potential candidates for further studies, we determined the expression of these integrins in various epithelial cell lines as shown in Figure 4. With the exception of integrin β6 all tested integrins showed robust expression at mRNA level in all cell lines. The expression of *β6* was weak in HEK293 (Figure 4C) and not detectable in Ad-AH cells (Figure 4E). All cell lines were subjected to direct infection by spinoculation with a recombinant wild type EBV strain encoding for GFP. We achieved the highest infection frequencies in AGS and HEK293 cells (see attached manuscript I; Figure 3) while the remaining cell lines were not or only weakly susceptible to infection by EBV (data not shown). Therefore, we chose these cell lines and additionally HONE-1 cells, as nasopharyngeal carcinoma model for our study as described in the attached manuscript I.

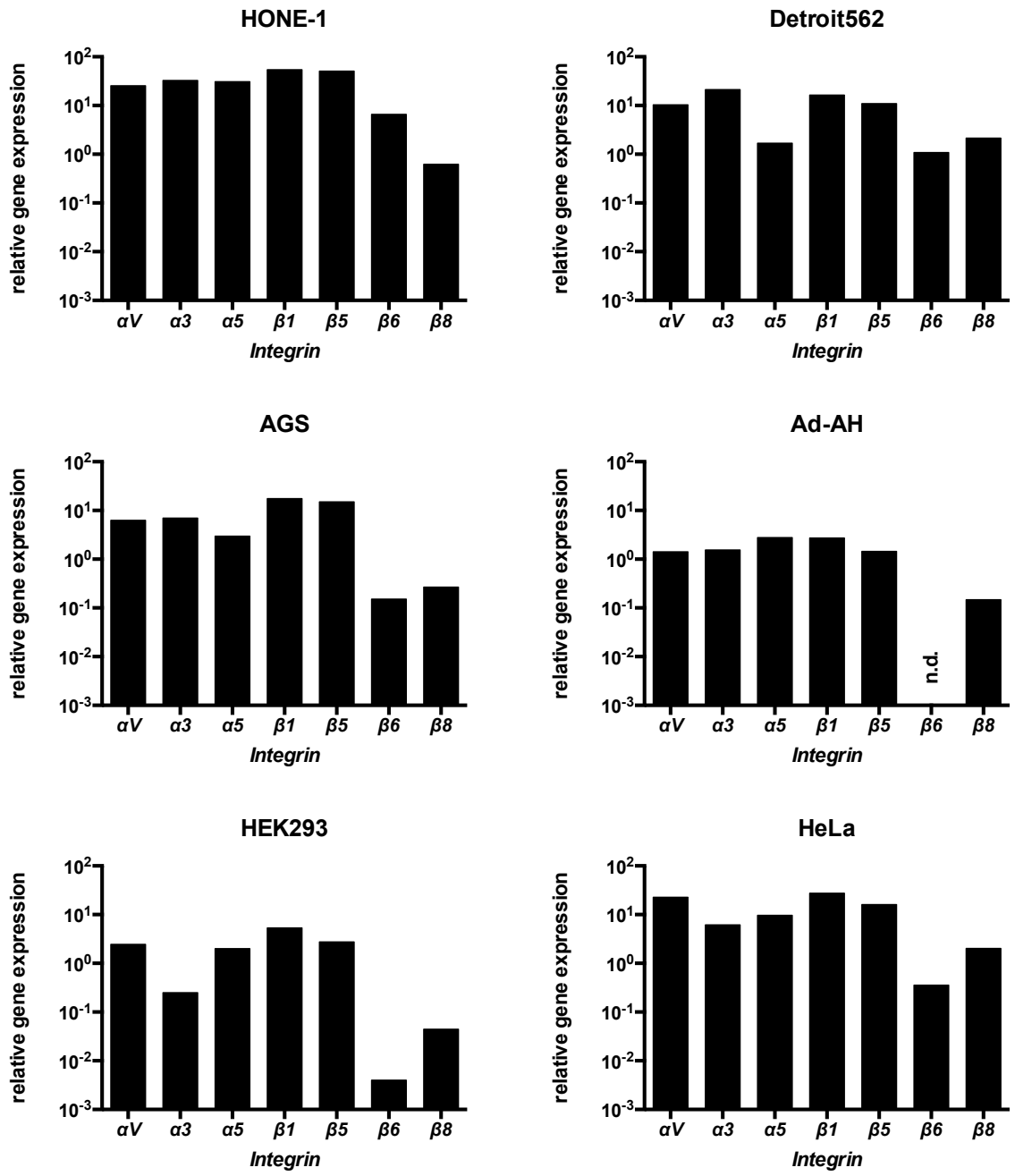

Figure 4: Integrin gene expression patterns in various epithelial cell lines. Integrin gene expression was determined in HONE-1 (A), AGS (B), HEK293 (C), Detroit562 (D), Ad-AH (E) and HeLa (F) cells by RT-qPCR relative to *HMBS*. Data is represented as Mean from 3 technical replicates of one experiment; n.d. = not detected.

Since hTERT-overexpressing cells showed at least in part increased infection frequencies as compared to the corresponding control cells (see attached manuscript I; Figure 3), we asked whether the expression of hTERT can regulate the expression of integrins, which are known to be involved in EBV attachment and entry in epithelial cells. We hypothesized that increased hTERT expression might up-regulate the expression of integrins and therefore lead to increased susceptibility to infection by EBV. We compared the integrin expression

pattern as described before, in wild type and hTERT-overexpressing HEK293 cells. As shown in Figure 5 we did not detect any significant difference that would lead to increased infection susceptibility. Unexpectedly, we found a loss of *integrin β6* expression in HEK293-hTERT cells. We conclude that hTERT is not involved in the regulation of integrin expression and that the differences seen in the infection frequencies might be not ascribed to increased susceptibility to infection by EBV.

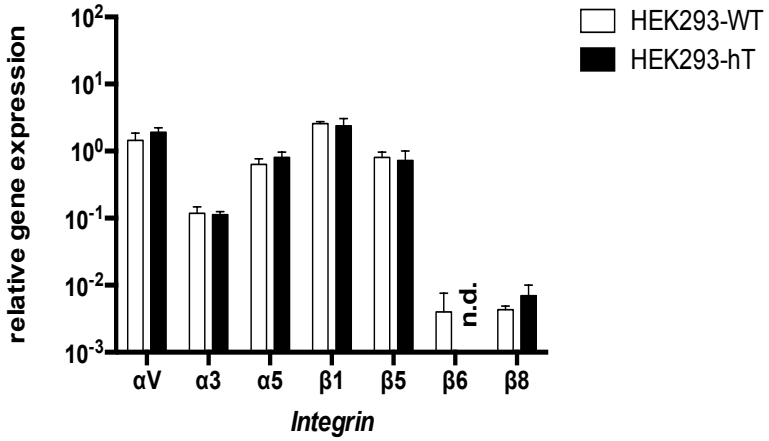

Figure 5: Integrin gene expression pattern in wild type and hTERT overexpressing HEK293 cells. Integrin gene expression was determined in wild type (HEK293-WT; white columns) and in hTERT overexpressing (HEK293-hT; black columns) HEK293 cells by RT-qPCR relative to *HMBS*. Data is represented as Mean ± SD from 3 independent experiments; n.d. = not detected.

Integrin expression patterns in primary sphenoidal epithelial cells

The susceptibility to infection by EBV was demonstrated by Feederle et al. to be dependent on the differentiation status of epithelial cells (164). Thereby, differentiated or late passage sphenoidal epithelial cells (sECs) showed a higher susceptibility to direct infection by EBV than less differentiated or early passage sECs. Since hTERT expression and telomerase activity is attributed to stem and progenitor cells within the basal layer of the epithelium we hypothesized that differentiated epithelial cells without hTERT expression and telomerase activity might show increased expression of integrins that contribute to infection of epithelial cells by EBV. To investigate the expression of the integrins, mentioned above, we received frozen cell pellets of sECs in early (passage number 2) and late (passage number 6-7) passages as a kind gift from Regina Feederle (German Cancer Research Center, Heidelberg, Germany). We isolated the total RNA, prepared cDNA and determined the gene expression levels by RT-qPCR relative to the housekeeping gene *HMBS*. We found no obvious

difference between early and late passage sECs regarding their integrin gene expression levels as shown in Figure 6. Nevertheless, late passage sECs showed slightly increased expression levels of all integrins tested except of β8. These results indicate that late passage epithelial cells might be more susceptible to EBV due to increased expression of integrins, known to be involved in the infection of epithelial cells. However, this appears to be in contrast with the well-known fact that integrin expression is found predominantly in the basal layers of multilayered epithelia. Differentiation and migration of epithelial cells to the apical surface of the epithelium is accompanied by a relative loss of integrin expression (223, 224). Additionally, we investigated the *hTERT* gene expression within early and late passage sECs. In total, we could detect *hTERT* gene expression only in 4 out of 9 samples at very low levels (relative gene expression $<10^{-2}$; data not shown). Whereas 1 early passage sEC (passage number 2), 2 intermediate passage sECs (passage number 4-5) and 1 of the late passage sECs (passage number 7) were positive for *hTERT*. These results additionally indicate that the susceptibility to infection by EBV is not dependent on hTERT expression.

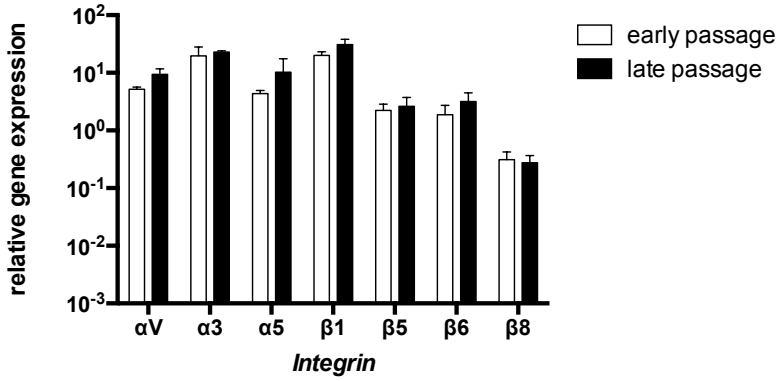

Figure 6: Integrin gene expression pattern in early and late passage sECs. Integrin gene expression was determined in early (white columns) and late (black columns) passage sECs by RT-qPCR relative to *HMBS*. Data is represented as Mean ± SD from 3 individual donors.

Growth properties of wild type and hTERT-overexpressing epithelial cell lines

Higher infection frequencies in hTERT-overexpressing cells as compared to control cells (see attached manuscript I; Figure 3) might be due to faster proliferation of cells with increased hTERT levels. Therefore, we investigated the proliferation characteristics of hTERT-overexpressing cells in comparison to the corresponding wild type cell lines by Trypan Blue exclusion and MTS assay as shown in Figure 7. Using the Trypan Blue exclusion assay, we did not observe any significant difference between wild type and hTERT-

overexpressing cells. Interestingly, we found slightly reduced proliferation rates in HONE-1-hTERT (Figure 7A) and AGS-hTERT (Figure 7B) cells in comparison to their corresponding wild type cells while the proliferation of HEK293-hTERT was similar to wild type HEK293 cells (Figure 7C). However, when we determined the proliferation capacity by MTS assay, HONE-1-hTERT cells showed comparable results to wild type HONE-1 cells (Figure 7D) whereas wild type AGS and AGS-hTERT cells (Figure 7E) showed similar results as compared to the corresponding Trypan Blue exclusion assay in Figure 7B. HEK293-hTERT cells showed significantly increased proliferation rates in comparison to wild type HEK293 cells when we used the MTS assay (Figure 7F). However, with the MTS assay one does not necessarily detect directly cell proliferation. The MTS assay is used to detect metabolic activity that is usually correlating with cell proliferation since proliferating cells are highly metabolically active. Since AGS-hTERT cells showed lower proliferation rates with both assays and HEK293-hTERT cells showed only higher proliferation rates using the MTS assay, we conclude that hTERT-overexpression does not substantially impact on the proliferation, which is in line with the findings of Hahn *et al.* (225), showing that increased expression of hTERT does not lead to enhanced proliferation in various cancer cell lines. Taken together, these results indicate that the proliferation capacity of hTERT-overexpressing cells does not account for the increased infection frequencies.

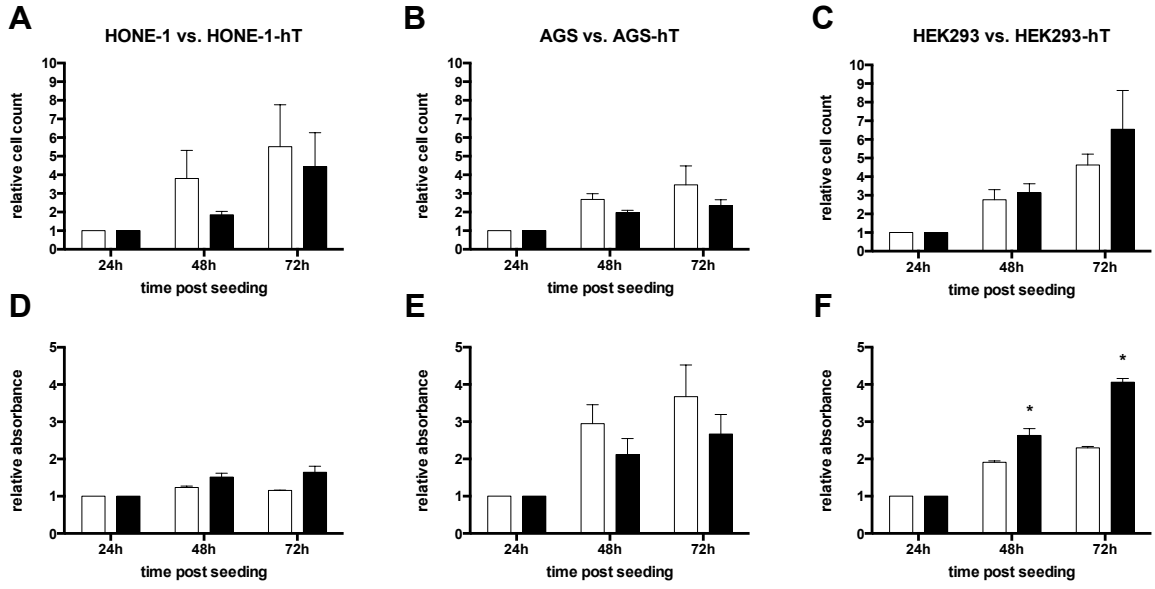

Figure 7: Proliferation characteristics of wild type and hTERT-overexpressing epithelial cell lines. Proliferation of wild type (white columns) and hTERT-overexpressing epithelial cell lines was determined by Trypan Blue exclusion assay (A-C) and by MTS assay (D-F) in HONE-1 vs. HONE-1-hTERT (A, D), AGS vs. AGS-hTERT (B, E) and HEK293 vs. HEK293-hTERT (C, F) cells. Data was normalized to the starting time point 24h and is represented as Mean ± SD of 3 independent experiments. * = $p<0.05$ (unpaired t test; Holm-Sidak method).

V.2 Manuscript II: TLR9 stimulation in epithelial cells does not drive Epstein-Barr virus into latency

Jürgen Rac, Florian Haas, Michele Bernasconi, Roberto F. Speck, David Nadal

Manuscript in preparation

V.2.1 Abstract

The ubiquitous γ-herpesvirus EBV is very successful in infecting >90% of the human population. EBV is transmitted via saliva to the next susceptible host and thus the pharyngeal cavities are portal of entry and exit for Epstein-Barr virus (EBV). Various microbes with specific pathogen associated molecular patterns (PAMPs) constantly challenge the oral mucosal epithelial tissues lining the pharyngeal cavities. As a first line of defense, the innate immune system recognizes these microorganisms via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Signaling triggered by TLR stimulation results in expression of pro-inflammatory cytokines, chemokines and anti-microbial effector molecules and is therefore contributing to shape the adaptive immunity as a second response to invading pathogens. Apart from the mechanical and physical barrier function of pharyngeal epithelial cells, they express TLRs and are capable of mounting an innate immune response. Thus epithelial cells of the pharyngeal cavity play a pivotal role not only within the life cycle of EBV. Recently we have demonstrated that TLR9 activation can suppress the reactivation from latent to lytic EBV infection in EBV positive Burkitt's lymphoma cells whereas triggering of TLR2 in latently infected lymphoblastoid cell lines elicits lytic EBV replication. However, the impact of activated TLR signaling on the infection of epithelial cells by EBV remains elusive. Therefore, we investigated the effect of TLR9 stimulation on EBV infection of epithelial cells. Here, we show that TLR9 triggering with synthetic ligands has no impact on EBV infection of the stably TLR9-expressing epithelial model cell line HEK293 and its wild type counterpart. Additionally, our results indicate that both cell lines might actually not be suitable as epithelial cell models to study the effect of TLR signaling on the infection of epithelial cells by EBV, but they suggest a contributing role of TLR activation in epithelial cells to EBV reactivation from latency.

For detailed information see attached Manuscript II.

V.2.2 Complementary Data and Results to Manuscript II

TLR expression patterns in various epithelial cell lines

TLRs play a central role in the innate immune defense mechanisms against invading pathogens, as already mentioned in the attached manuscript II. Since EBV is an orally transmitted virus, it first encounters the epithelial cells within the pharyngeal cavity before EBV gets access to its preferred target, the B cells. Epithelial cells within the pharyngeal and sinonasal cavities are known to express TLRs, whereas mainly TLR2, 3 and 4 seem to be important in primary epithelial cells and cell lines from the pharynx. Nevertheless, we recently demonstrated that TLR9 triggering can inhibit lytic EBV replication upon primary infection of B cells and suppresses the switch from latent tor lytic EBV in latently infected BL cells *in vitro* (211, 212). By contrast, TLR2 activation using heat-killed GAS elicited lytic EBV replication in latently infected LCLs established from TMCs (213). This goes along with the finding that GAS colonized patients shed substantially higher numbers of EBV particles into saliva (213). However, the impact of TLR activation on the infection of epithelial cells by EBV remains elusive and we decided to investigate the role of TLR9 activation on EBV infection of epithelial cells. Therefore, we screened several epithelial cell lines for their TLR gene expression patterns as shown in Figure 8. It turned out that the TLR gene expression pattern is quite heterogeneous amongst the tested cell lines. While we detected expression of TLR1, 3 and 6 in all cell lines, including HEK293 (see attached manuscript II; Figure 1), the expression of the remaining TLRs was inconsistent. However, the nasopharyngeal carcinoma cell line Detroit562 (Figure 8C) was the only cell line that expressed all TLR, characteristic for pharyngeal epithelial cells. Since HEK293 was the only cell line with detectably levels of TLR9, apart from HONE-1 and AGS, and had the highest susceptibility direct infection by EBV we chose the cell line HEK293 for our study as described in the attached manuscript II.

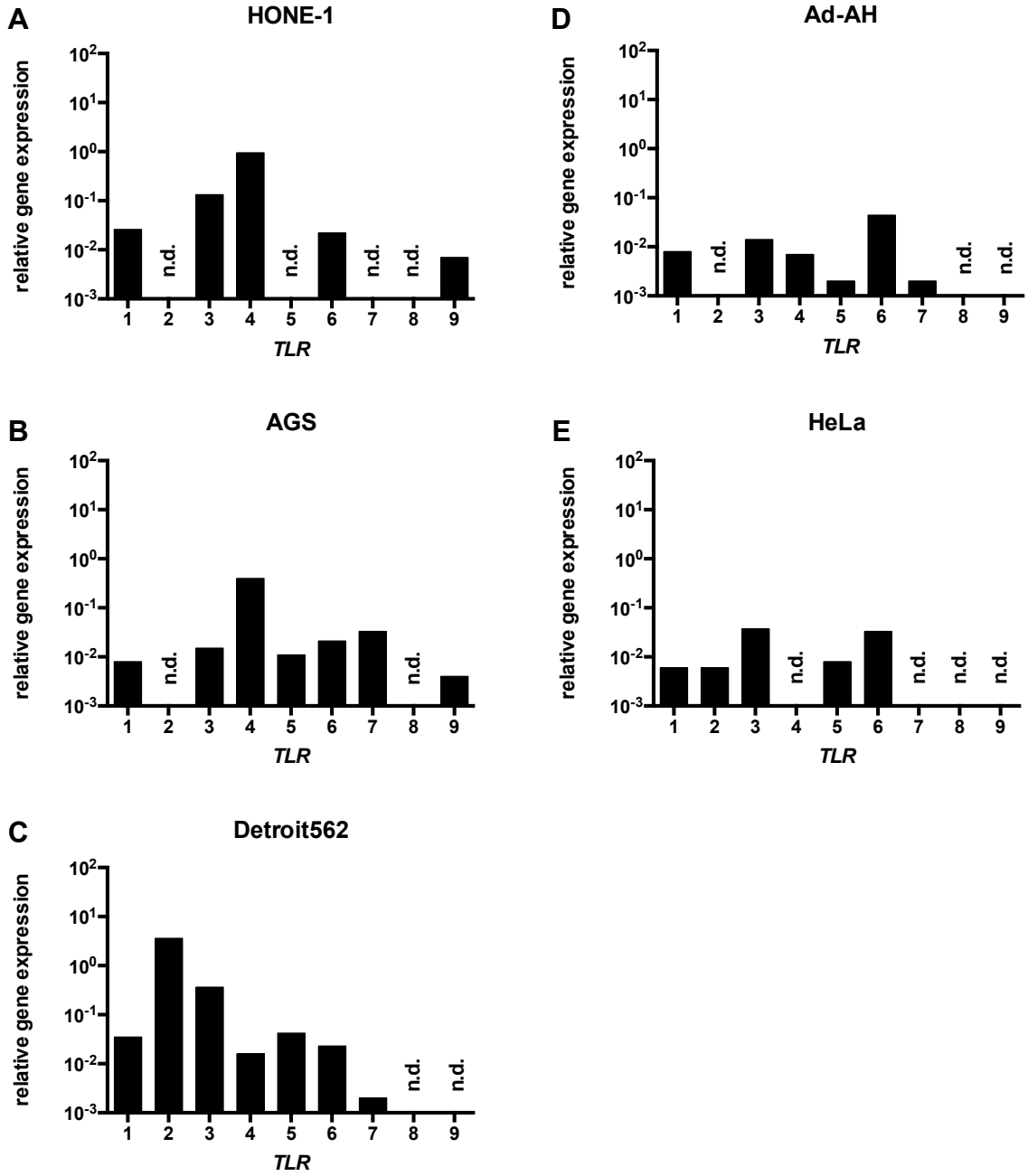

Figure 8: TLR gene expression patterns in various epithelial cell lines TLR gene expression was determined in HONE-1 (A), AGS (B), Detroit562 (C), Ad-AH (D) and HeLa (E) cells by RT-qPCR relative to *HMBS*. Data is represented as Mean from 3 technical replicates of one experiment; n.d. = not detected.

TLR expression patterns in primary sphenoidal epithelial cells

In addition to the investigation of the TLR gene expression patterns in the epithelial model cell lines, we determined the TLR gene expression in the primary sECs (Figure 9). In general, these cells showed almost the same expression pattern like the nasopharyngeal cell line Detroit562 (Figure 8C). The only difference was the lack of *TLR7* expression, which was present in Detroit562 cells, and the expression of *TLR9*, which was absent in Detroit562 cells. Since we did not observe any significant effect of TLR9 activation on EBV infection of wild type and TLR9-expressing HEK293 cells, these results suggest that primary epithelial cells such as sECs and tonsillar epithelial cells, might be more suitable models to test the impact of TLR activation on the infection of epithelial cells. Contributing to this finding we compared as well the TLR expression in early and late passage sECs. Since late passage sECs reflect more differentiated cells, potentially located at the apical epithelial surface and therefore more frequently exposed to invading microorganisms, we hypothesized that these cells might express higher TLR levels compared to early passage or less differentiated sECs. This would render them potentially more sensitive to pathogens and evoke a stronger innate immune response. Although we did not detect any significant difference between early and late passage sECs, we observed at least a slightly increased expression of *TLR1*, *2*, *3*, *4* and *5* in late passage sECs (Figure 9). Taken together, these results indicate that primary sECs and especially differentiated cells might provide a suitable model to study the impact of TLR activation on the infection of epithelial cells by EBV.

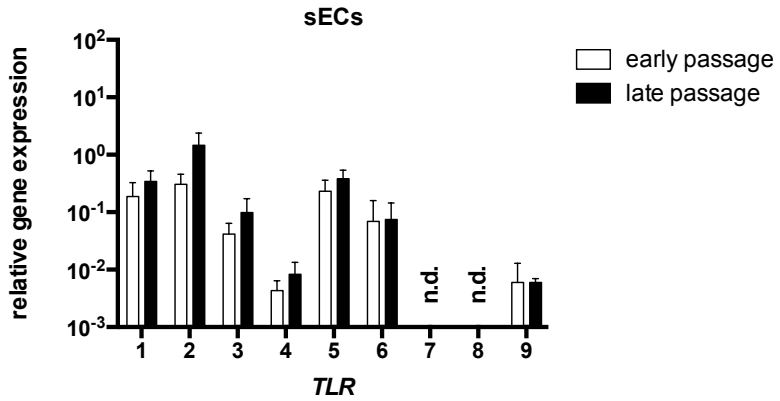

Figure 9: TLR gene expression patterns in early and late passage sECs. TLR gene expression was determined in early (white columns) and late (black columns) passage sECs by RT-qPCR relative to *HMBS*. Data is represented as Mean ± SD from 3 individual donors; n.d. = not detected.

VI COMPLEMENTARY MATERIAL AND METHODS

For detailed descriptions of materials and methods that are not mentioned within this section, please refer to the attached manuscripts I & II.

Cells

As additional model epithelial cell lines we used the nasopharyngeal carcinoma (NPC) cell line Detroit562 (226, 227), maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Buchs, Switzerland), the cervical adenocarcinoma cell line HeLa (228), maintained in RPMI-1640 (Sigma-Aldrich), and the nasopharyngeal cell line Ad-AH (229), kindly provided by Claire Shannon-Lowe (University of Birmingham, Birmingham, United Kingdom), as well maintained in RPMI-1640. All media were supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Sigma-Aldrich), 1% L-Glutamine and 1% Penicillin/Streptomycin (Gibco, Zug, Switzerland). Frozen cell pellets sphenoidal sinus epithelial cells (sECs) were kind gifts from Regina Feederle and Henri-Jacques Delecluse (German Cancer Research Center, Heidelberg, Germany) (164).

Gene expression analysis by RT-qPCR

Integrin gene expression was determined using a pre-validated primer/probe assays for integrin αV (Hs00233808), α5 (Hs01547673), α3 (Hs00233722), β1 (Hs00559595), β3 (Hs01001469), β5 (Hs00174435), β6 (Hs00168458) and β8 (Hs01110394) (all from Applied Biosystems; Zug, Switzerland). For detailed description of RNA isolation, cDNA preparation, RT-qPCR reactions, calculation and analysis of the data please refer to the attached manuscripts I & II.

Cell proliferation analysis

Proliferation of wild type and hTERT-overexpressing epithelial cells was determined by Trypan Blue exclusion and a colorimetric (MTS) assay. Briefly, for the Trypan Blue exclusion assay, $5x10^4$ cells/well were seeded in 24-well plates (TPP, Trasadingen, Switzerland) and incubated at 37°C with 5% CO2. At 24 h, 48 h and 72 h post seeding cells were detached, using 0.25% Trypsin-EDTA (Gibco, Zug, Switzerland), washed with 1x phosphate buffered saline (PBS; Gibco, Zug, Switzerland), resuspended in 1 ml 1x PBS and 10 µl cell suspension were mixed with 10 µl 0.4% Trypan Blue dye (Gibco). The mix was

transferred to a hemocytometer and unstained (live) cells were counted. The total number of live cells was calculated on the basis of the cell suspension volume (1 ml 1x PBS) and the dilution factor (1:2) of the mix. Calculated total number of live cells was normalized to the starting time point at 24h post seeding. All cell lines were counted at least in duplicate for each experiment.

For the MTS assay, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Dübendorf, Switzerland) was used according to manufacturer's instructions. Briefly, $5x10^3$ cells/well were seeded on 96-well flat bottom plates (Sarstedt, Sevelen, Switzerland) and incubated at 37°C with 5% CO2. At 24 h, 48 h and 72 h post seeding, 20 µl/well CellTiter 96® AQ_{ueous} One Solution Reagent (containing the tetrazolium compound MTS) were added, incubated for 2 h at 37°C and the absorbance at 490 nm was determined using the Synergy HT plate reader (BioTek, Luzern, Switzerland). Medium alone served as blank control to determined the background absorbance that was subtracted from the signal obtained for each sample. The absorbance was normalized to the starting time point at 24 h post seeding. All reactions were carried out in triplicates for each experiment.

VII GENERAL DISCUSSION AND OUTLOOK

The B-lymphotropic γ-herpesvirus EBV is transmitted via saliva to the next naïve host. Thus, the portal of entry and exit for EBV are the pharyngeal cavities that are in addition constantly challenged by various microbes. The mucosal epithelia lining the oro- and nasopharynx are therefore the first and the last barrier EBV has to overcome to get access to its preferred target cell, the B cell. Thus, epithelial cells play a pivotal role within the life cycle of EBV and it is not surprising that EBV is as well associated with epithelial cell malignancies like NPC and GC. Within this work I performed studies on two different aspects of the EBV infection in epithelial cells. First, we investigated the contribution of hTERT, the rate-limiting component of the telomerase, on EBV infection in epithelial cells (see attached manuscript I). Second, we studied the impact of TLR9 activation on the infection of epithelial cells by EBV (see attached manuscript II). The obtained results will be discussed within this section with regard to the putative role of the epithelium as transit route for EBV during its the life cycle.

EBV infection *in vivo*

While the infection of B cells by EBV has been studied extensively and is quite well understood, the infection biology and the role of epithelial cells within the life cycle of EBV still remains puzzling and mostly elusive. This is partly due to the lack of suitable *in vitro* epithelial cell infection models for EBV. Soon after the isolation of EBV, it has been demonstrated that EBV is shed within the pharyngeal cavities into saliva and oropharyngeal epithelial cells were considered to be the potential source of the progeny virus for transmission (230–234). *In vivo*, EBV is only found in a latent state in EBV-associated epithelial malignancies such as NPC and EBVaGC as discussed in the introduction. Lytic EBV replication was found to occur in the context of oral hairy leukoplakia (OHL), an acquired immunodeficiency syndrome (AIDS)-associated epithelial lesion at the lateral margin of the tongue (235–239) and further evidence suggests that reactivation of lytic EBV infection most commonly occurs in tonsillar plasma cells as well as in tonsillar B-cells (184, 240–242). In addition, it has been difficult or even impossible to find any evidence of lytically infected epithelial cells in immunocompetent individuals (181, 182). Reactivation and lytic replication of EBV in B cells alone cannot account for the large amounts of virus found in saliva (100). This finding is further supported by the observation of oropharyngeal EBV

shedding in the absence of circulating B cells in patients treated with the anticancer drug rituximab (243). However, the treatment with rituximab does not entirely eliminate lymphocytes from lymphoid tissues (244, 245), as these are present in NALT. Taken together, the cellular source of progeny virus for transmission is a matter of debate and still needs to be identified. Speculatively, it might be a cooperation of both, reactivation of EBV in B lymphocytes and lytic EBV infection of epithelial cells, e.g. within the lymhoepitelium of the NALT where B cells and epithelial cells are in close proximity to each other. In support of this theory comes the observation that EBV can lytically replicate in tonsillar plasma cells (184). In conclusion, these findings imply more a role as transit route or the epithelium within the life cycle of EBV as already suggested by others (160, 240).

EBV infection *in vitro*

The failure to detect EBV-infected epithelial cells *in vivo*, apart from latently infected tumor cells and the tongue lesion in OHL, is clearly reflected *in vitro*. The *in vitro* infection of epithelial using cell-free EBV is difficult to achieve and not efficient (162). However, several infection mechanisms have been proposed and demonstrated, as mentioned in the introduction. One major breakthrough in the understanding of the infection biology of EBV in epithelial cells was considered the investigation of cell-to-cell contact infection, using either EBV-infected B cells or EBV-loaded B cells as transfer vehicle (25). However, the findings are controversial. While Tugizov *et al.* reported efficient infection of polarized nasopharyngeal epithelial cells using infected lymphoblastoid cell lines from the apical surface (153), Shannon-Lowe *et al.* reported efficient and rapid transfer infection using EBV-loaded primary B cells from the basolateral surface of primary tonsillar epithelial cells (167). This suggests differences between cell-to-cell contact and B cell-mediated transfer infections. However, both approaches generate EBV particles more specific for epithelial cells, as predicted by the concept of switch in EBV cell tropism (143). However, epithelial cells in the upper apical epithelial layers lack expression of integrins that potentially serve as EBV receptors. Speculatively, this suggests that cell-to-cell contact-mediated infection of epithelial cells from the apical surface occurs *via* membrane fusion without release of EBV from B cells. By contrast, reactivation of EBV in B cells, release of EBV and subsequent binding of EBV to B cells would be needed for B cell-mediated transfer infection of epithelial cells from the basal surface. Following the hypothesis, attachment of EBV to the B cells would lead to conformational changes within the viral envelope, thus giving rise to the viral ligands for the epithelial receptors, e.g. integrins, at the basal surface of epithelial cells as mentioned in the

introduction. On the other hand, fusion of B cells and epithelial cells might potentially occur *in vivo* from the basal surface as well. Membrane fusion was demonstrated in *in vitro* assays *via* the viral glycoprotein complex gHgL and the cellular integrins αVβ5, αVβ6 and αVβ8 (157–159). B cell-mediated transfer infections from the basal epithelial surface might be biologically more relevant in the *in vivo* situation since the presence of B cells in saliva is probably very rare. Notwithstanding, the exact mechanisms of cell-to-cell contact and B cell-mediated transfer infections remain to be elucidated. Nevertheless, EBV might employ both mechanisms *in vivo* in addition to cell-free infection mechanisms at different steps during its life cycle. The infection of epithelial cells with cell-free EBV in general is difficult to achieve *in vitro,* as mentioned above. Nevertheless, infection of polarized nasopharyngeal epithelial cells was reported to be more efficient from the basolateral surface (153, 155). Additionally, EBV was demonstrated to be bi-directionally transcytosed through polarized primary tonsillar epithelial cells (160). Moreover, basal to apical transcytosis might be facilitated *in vivo* through EBV-specific IgA molecules (147, 148), whereas gp350/220-specific IgG molecules in saliva might facilitate EBV entry into the epithelium from the apical surface (143, 166). The finding that EBV in the saliva contains higher levels of gp42 (246) indicates that EBV was produced in epithelial cells and further contributes to the hypothesis that EBV might directly infect B cells residing in the epithelium. Thus supporting the possibility that transmitted EBV could initially bypass the epithelium, without productive infection of epithelial cells, either by transcytosis or within infected B cells before reaching the host B cell pool and persisting life-long.

The epithelium and its potential role as transit route for EBV

Taken together, the role for the epithelium within the life cycle of EBV is ambiguous. It seems that most of the strategies used by EBV to overcome the epithelial barrier are more or less of unspecific nature. That's probably because we still do not completely understand them. Nonetheless, EBV is able to infect epithelial cells *via* various mechanisms or can be transported through the epithelium without a productive infection of epithelial cells. Therefore, I agree with the proposed role as transit route for the epithelium in the life cycle of EBV. Moreover, from my point of view, the infection of susceptible epithelial cells by EBV within the basal epithelial layers occurs rather accidently, while the interplay between B cells and epithelial cells in the upper epithelial layers (more differentiated) might be necessary for virus amplification. The known mechanisms how EBV may overcome the epithelia within its putative portal of entry and exit, the pharyngeal cavities, are summarized in Figure 10.

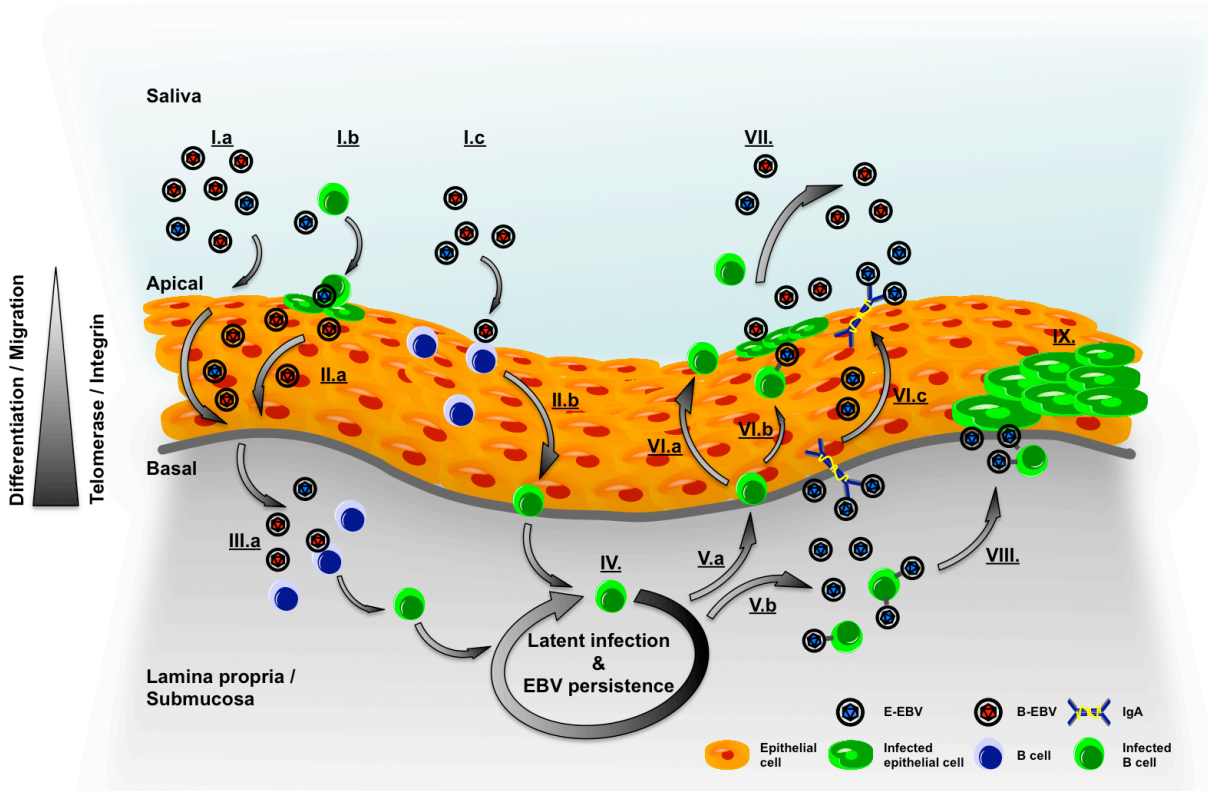

Figure 10: Model for epithelium as transit station within the life cycle of EBV. Mainly EBV with increased B cell tropism (B-EBV, red) is transmitted *via* saliva as cell-free virions (I.a/c), or within infected B cells that directly lytically infect differentiated epithelial cells by cell-to-cell contact (I.b). Cell-free B-EBV might directly attach and enter B cells that reside within the epithelium (I.c), e.g. in the lymphoepithelia of the nasopharynx-associated lymphoid tissue (NALT). B-EBV is then transcytosed through the epithelial layers either as cell-free virus (II.a). Whereas EBV establishes a latent infection upon entry into the epithelia-associated B cells, which then translocate/transmigrate through the epithelium (II.b). Transcytosed B-EBV then infects B cells located in the *lamina propria* and *submucosa* (III.a). EBV-infected B cells (III.a/b) subsequently enter the B cell-associated part of the EBV life cycle (IV.), where EBV persists life-long in a latent state within the host B cell pool. Latently infected B cells might leave the B cell pool again (V.a) or lytically reactivate where progeny EBV with increased epithelial tropism (E-EBV, blue) is produced (V.b). Latently infected B cells might as well again translocate/transmigrate through the epithelium (VI.a). In addition, EBV might reactivate in latently infected B cells within the epithelium, which then transfer E-EBV to differentiated epithelial cells in the upper layers of the epithelium where lytic EBV replication takes place (VI.b). Together, this leads to EBV amplification and production mainly of B-EBV for transmission. Additionally, cell-free E-EBV might be transcytosed through the epithelium, which could be facilitated by EBV-specific IgA molecules (VI.c). Finally, latently infected B cells and cell-free EBV are released into saliva and transmitted to the next susceptible host (VII.). Apart from EBV transmission, susceptible epithelial cells within the basal epithelial layer might be infected by cell-free E-EBV or by B cell-mediated E-EBV transfer (VIII.), which might lead to latent infection and clonal outgrowth of epithelial cells, thus ultimately to the development of tumors (IX.) such as NPC.

Telomerase and its impact on the EBV infection in epithelial cells

Telomerase expression and activity is usually restricted to continuously proliferating cells such as stem cells and it is as well a hallmark of cancer (125, 126). Epithelial stem cells with self-renewal capacity and indefinite proliferation capability are located within the basal layers of epithelial tissues (127–132). Since EBV has to bypass the epithelium, either to get access to the B cells or to be released and transmitted, it is likely that EBV comes into contact

with these epithelial stem cells. Due to EBV's tight association with epithelial malignancies such as NPC and EBVaGC we investigated the impact of hTERT on the EBV infection in epithelial cells (see manuscript I). Therefore we generated epithelial model cell lines with either increased hTERT expression levels or overexpression of a catalytically inactive dominant negative mutant DNhTERT. We found that increased telomerase expression and activity led to increased infection frequencies in the EBV susceptible, hTERT-overexpressing (hT) cell lines AGS-hT and HEK293-hT. In contrast, suppression of telomerase activity by ectopic expression of DNhTERT (DN) resulted in reduced infection frequencies at least in AGS-DN cells. Additionally, we showed that the EBV infection of epithelial cells is strongly dependent on the cellular context. Interestingly, increased hTERT expression and telomerase activity led to up-regulation mainly of latency-associated EBV genes. We therefore concluded that increased telomerase expression and activity contributes to EBV maintenance and in turn might facilitate tumor development and progression. It remains unclear whether cells with increased hTERT expression are more susceptible to an infection by EBV or if cells with reduced telomerase activity are not able to maintain the virus and therefore show lower infection frequencies. Nevertheless, our results are complementary to the observation that telomerase-immortalized primary nasopharyngeal cell clones could support a long-term EBV infection *in vitro* (60, 185). However, we did not find induction of lytic EBV replication due to suppression of telomerase expression and activity as demonstrated in B cells (187, 188). In general, the results of this study contribute to the hypothesis that EBV infection is a late or secondary event within the development of NPC and EBVaGC since latent and persistent EBV infection in epithelial cells is obviously dependent on cellular alteration such as loss of p16 activity and cyclin D1 overexpression (25). Such cellular alterations might be acquired by epithelial stem cells due to their self-renewal capacity and their proliferation capability. Moreover, EBV might activate telomerase activity *via* LMP1 since it was demonstrated that LMP1 can activate the hTERT promoter in B cells and epithelial cells (82, 124). Interestingly, LMP1 showed the strongest up-regulation in our hTERT-overexpressing model cell lines, indicating that telomerase activity and LMP1 expression might cooperate within a positive feedback loop. Taken together, our results support the role of EBV as a late event in NPC and EBVaGC and the latent EBV infection in combination with increased telomerase activity might facilitate tumor development and might be responsible for rapid tumor progression.

TLR activation and its impact on the infection of epithelial cells by EBV

Our second study focused on the influence of the innate immune mechanisms on the EBV infection in epithelial cells. Since various microbes constantly challenge the pharyngeal cavities, as portal of entry and exit for EBV, we hypothesized that an activated innate immune system might have an influence on the EBV infection in epithelial cells. More precisely, we tested the impact of TLR9 stimulation (see manuscript II). As part of the innate immune system, TLRs play a pivotal role within the first line of defense against invading pathogens. Activated TLR signaling ultimately leads to the expression of anti-microbial effector molecules such as pro-inflammatory cytokines, chemokines and type I interferons (198) and thus TLRs are crucial for preventing infections of the host. Upon TLR stimulation different scenarios can be imagined. First, TLR activation in epithelial cells might either block or facilitate EBV infection of epithelial cells. Second, activated TLR signaling by chronic stimulation might facilitate a latent EBV infection, leading expansion and malignant outgrowth of infected cells. Third, stimulation of TLRs in epithelial cells might serve as alarm signal and trigger lytic EBV replication and amplification, thus facilitating EBV transmission. However, in our *in vitro* model system we did not observe any significant effect of activated TLR9 signaling on the infection of the used epithelial model cells. Activation of TLR9 with two different synthetic TLR9 ligands had no impact on the infection frequencies of the tested epithelial model cell lines. Additionally, EBV gene expression did not show any significant changes due to TLR9 activation. It is possible that the employed epithelial model cell lines do not reflect the physiological *in vivo* situation to study the impact of TLR activation on the EBV infection in epithelial cells. Nevertheless, our group has recently shown that TLR9 triggering can inhibit the switch from latent to lytic EBV upon *de novo in vitro* infection of cord blood B cells (211) and upon B cell receptor (BCR)-induced reactivation of EBV in chronically infected BL cell lines (212). In addition we demonstrated that TLR2 ligation could induce lytic EBV reactivation in latently infected LCLs (213), whereas others have shown that activation of TLR2, 3 and 4 in BL cell lines led to up-regulation of lytic EBV genes (214). Taken together, these studies show that TLR signaling has an impact on EBV infection in general or at least in B cells. Nevertheless, these effects seem to be dependent on various factors, e.g. the physiological site of infection, the expressed TLR and potentially the infected cell type.

Future perspectives

In summary, the main findings of the work presented here show that EBV might benefit from the infection of epithelial cells with increased telomerase expression and activity, while the impact of activated TLR signalling on the EBV infection in epithelial cells remains elusive. Further efforts should focus on the establishment and the study of primary epithelial cell cultures. especially on polarized epithelial cell, epithelial stem cell and tissue cultures and the mechanisms how EBV might overcome theses barriers with regard to transcytosis of EBV as for example demonstrated by Tugizov *et al.* (160). Additionally, variations amongs EBV strains should be considered for further studies on the infection biology of EBV in epithelial cells since there are obviously EBV strains with markedly increased epithelial tropism as shown by Tsai *et al.* (165). Such studies might facilitate the understanding of EBV and its relation to epithelial cells and associated malignancies.

VIII REFERENCES

1. **Kieff ED, Rickinson AB**. 2007. Epstein-Barr Virus and Its Replication, p. 2603–2654. *In* Knipe, DM, Howley, PM (eds.), Fields Virology, 5th ed. Lippincott Williams & Wilkins.
2. **Luzuriaga K, Sullivan JL**. 2010. Infectious mononucleosis. N. Engl. J. Med. **362**:1993–2000.
3. **Henle G, Henle W, Clifford P, Diehl V, Kafuko GW, Kirya BG, Klein G, Morrow RH, Munube GM, Pike P, Tukei PM, Ziegler JL**. 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J. Natl. Cancer Inst. **43**:1147–57.
4. **Balfour HH, Odumade O a, Schmeling DO, Mullan BD, Ed J a, Knight J a, Vezina HE, Thomas W, Hogquist K a**. 2013. Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. J. Infect. Dis. **207**:80–8.
5. **Rickinson AB, Kieff ED**. 2007. Epstein-Barr Virus, p. 2656–2700. *In* Knipe, DM, Howley, PM (eds.), Fields Virology, 5th ed. Lippincott Williams & Wilkins.
6. **Babcock GJ, Decker LL, Volk M, Thorley-Lawson D a**. 1998. EBV persistence in memory B cells in vivo. Immunity **9**:395–404.
7. **Kenney SC, Mertz JE**. 2014. Regulation of the latent-lytic switch in Epstein-Barr virus. Semin. Cancer Biol. 1–9.
8. **Griffin BD, Verweij MC, Wiertz EJHJ**. 2010. Herpesviruses and immunity: the art of evasion. Vet. Microbiol. **143**:89–100.
9. **Hislop AD, Taylor GS, Sauce D, Rickinson AB**. 2007. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu. Rev. Immunol. **25**:587–617.
10. **Speck SH, Ganem D**. 2010. Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe **8**:100–15.
11. **Taylor GS, Blackbourn DJ**. 2011. Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV. Cancer Lett. **305**:263–78.
12. **Grinde B**. 2013. Herpesviruses: latency and reactivation - viral strategies and host response. J. Oral Microbiol. **5**:1–9.
13. **Epstein MA, Achong BG, Barr YM**. 1964. Virus particles in cultured lymphoblasts from Burkitt's Lymphoma. Lancet **1**:702–3.
14. **Henle W, Henle G**. 1973. Evidence for an oncogenic potential of the Epstein-Barr virus. Cancer Res. **33**:1419–23.
15. **Pagano JS, Blaser M, Buendia M-A, Damania B, Khalili K, Raab-Traub N, Roizman B**. 2004. Infectious agents and cancer: criteria for a causal relation. Semin. Cancer Biol. **14**:453–71.
16. **Molyneux EM, Rochford R, Griffin B, Newton R, Jackson G, Menon G, Harrison CJ, Israels T, Bailey S**. 2012. Burkitt's lymphoma. Lancet **379**:1234–44.
17. **Ambinder RF**. 2007. Epstein-barr virus and hodgkin lymphoma. Hematology Am. Soc. Hematol. Educ. Program 204–9.
18. **Weiss LM, Strickler JG, Warnke RA, Purtilo DT, Sklar J**. 1987. Epstein-Barr viral DNA in tissues of Hodgkin's disease. Am. J. Pathol. **129**:86–91.
19. **Nourse JP, Jones K, Gandhi MK**. 2011. Epstein-Barr Virus-related post-transplant lymphoproliferative disorders: pathogenetic insights for targeted therapy. Am. J. Transplant **11**:888–95.
20. **Gottschalk S, Rooney CM, Heslop HE**. 2005. Post-transplant lymphoproliferative disorders. Annu. Rev. Med. **56**:29–44.
21. **Dolcetti R**. 2007. B lymphocytes and Epstein-Barr virus: the lesson of post-transplant lymphoproliferative disorders. Autoimmun. Rev. **7**:96–101.
22. **Taylor AL, Marcus R, Bradley JA**. 2005. Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. Crit. Rev. Oncol. Hematol. **56**:155–67.

23. **Gu A-D, Zeng M-S, Qian C-N**. 2012. The criteria to confirm the role of epstein-barr virus in nasopharyngeal carcinoma initiation. Int. J. Mol. Sci. **13**:13737–47.

24. **Lo KW, To KF, Huang DP**. 2004. Focus on nasopharyngeal carcinoma. Cancer Cell **5**:423–8.

25. **Tsao SW, Tsang CM, Pang PS, Zhang G, Chen H, Lo KW**. 2012. The biology of EBV infection in human epithelial cells. Semin. Cancer Biol. **22**:137–43.

26. **Lo K-W, Chung GT-Y, To K-F**. 2012. Deciphering the molecular genetic basis of NPC through molecular, cytogenetic, and epigenetic approaches. Semin. Cancer Biol. **22**:79–86.

27. **Shah KM, Young LS**. 2009. Epstein-Barr virus and carcinogenesis: beyond Burkitt's lymphoma. Clin. Microbiol. Infect. **15**:982–988.

28. **Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N**. 1995. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N. Engl. J. Med. **333**:693–8.

29. **Young LS, Dawson CW, Clark D, Rupani H, Busson P, Tursz T, Johnson A, Rickinson AB**. 1988. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J. Gen. Virol. **69 (Pt 5)**:1051–65.

30. **Pathmanathan R, Prasad U, Chandrika G, Sadler R, Flynn K, Raab-Traub N**. 1995. Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia. Am. J. Pathol. **146**:1355–67.

31. **Iizasa H, Nanbo A, Nishikawa J, Jinushi M, Yoshiyama H**. 2012. Epstein-Barr Virus (EBV)-associated gastric carcinoma. Viruses **4**:3420–39.

32. **Zur Hausen A, van Rees BP, van Beek J, Craanen ME, Bloemena E, Offerhaus GJA, Meijer CJLM, van den Brule AJC**. 2004. Epstein-Barr virus in gastric carcinomas and gastric stump carcinomas: a late event in gastric carcinogenesis. J. Clin. Pathol. **57**:487–91.

33. **Fukayama M, Ushiku T**. 2011. Epstein-Barr virus-associated gastric carcinoma. Pathol. Res. Pract. **207**:529–37.

34. **Gulley ML, Pulitzer DR, Eagan P a, Schneider BG**. 1996. Epstein-Barr virus infection is an early event in gastric carcinogenesis and is independent of bcl-2 expression and p53 accumulation. Hum. Pathol. **27**:20–7.

35. **Levine PH, Stemmermann G, Lennette ET, Hildesheim A, Shibata D, Nomura A**. 1995. Elevated antibody titers to Epstein-Barr virus prior to the diagnosis of Epstein-Barr-virus-associated gastric adenocarcinoma. Int. J. Cancer **60**:642–4.

36. **Kutok JL, Wang F**. 2006. Spectrum of Epstein-Barr virus-associated diseases. Annu. Rev. Pathol. **1**:375–404.

37. **Young LS, Sung NS, S PJ**. 2009. Epstein–Barr Virus. Encycl. Life Sci. John Wiley & Sons, Ltd, Chichester, UK.

38. **Yu MC, Yuan J**. 2002. Epidemiology of nasopharyngeal carcinoma. Semin. Cancer Biol. **12**:421–9.

39. **Akiba S, Koriyama C, Herrera-Goepfert R, Eizuru Y**. 2008. Epstein-Barr virus associated gastric carcinoma: epidemiological and clinicopathological features. Cancer Sci. **99**:195–201.

40. **Chen J-N, He D, Tang F, Shao C-K**. 2012. Epstein-Barr virus-associated gastric carcinoma: a newly defined entity. J. Clin. Gastroenterol. **46**:262–71.

41. **Dawson CW, Port RJ, Young LS**. 2012. The role of the EBV-encoded latent membrane proteins LMP1 and LMP2 in the pathogenesis of nasopharyngeal carcinoma (NPC). Semin. Cancer Biol. **22**:144–53.

42. **Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, Tanaka S, Sato E, Osato T**. 1994. Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proc. Natl. Acad. Sci. U. S. A. **91**:9131–5.

43. **Minarovits J, Hu LF, Imai S, Harabuchi Y, Kataura A, Minarovits-Kormuta S, Osato T, Klein G**. 1994. Clonality, expression and methylation patterns of the Epstein-Barr virus genomes in lethal midline granulomas classified as peripheral angiocentric T cell lymphomas. J. Gen. Virol. **75 (Pt 1)**:77–84.

44. **Deyrup AT**. 2008. Epstein-Barr virus-associated epithelial and mesenchymal neoplasms. Hum. Pathol. **39**:473–83.

45. **Shanmugaratnam K**. 1978. Histological typing of nasopharyngeal carcinoma. IARC Sci. Publ. 3–12.

46. **Parkin DM, Bray F, Ferlay J, Pisani P**. Global cancer statistics, 2002. CA. Cancer J. Clin. **55**:74–108.

47. **Lee AWM, Foo W, Mang O, Sze WM, Chappell R, Lau WH, Ko WM**. 2003. Changing epidemiology of nasopharyngeal carcinoma in Hong Kong over a 20-year period (1980-99): an encouraging reduction in both incidence and mortality. Int. J. Cancer **103**:680–5.

48. **Nielsen NH, Mikkelsen F, Hansen JP**. 1977. Nasopharyngeal cancer in Greenland. The incidence in an Arctic Eskimo population. Acta Pathol. Microbiol. Scand. A. **85**:850–8.

49. **Buell P**. 1974. The effect of migration on the risk of nasopharyngeal cancer among Chinese. Cancer Res. **34**:1189–91.

50. **Dickson RI, Flores AD**. 1985. Nasopharyngeal carcinoma: an evaluation of 134 patients treated between 1971-1980. Laryngoscope **95**:276–83.

51. **Herath CHP, Chetty R**. 2008. Epstein-Barr virus-associated lymphoepithelioma-like gastric carcinoma. Arch. Pathol. Lab. Med. **132**:706–9.

52. **Uozaki H, Fukayama M**. 2008. Epstein-Barr virus and gastric carcinoma--viral carcinogenesis through epigenetic mechanisms. Int. J. Clin. Exp. Pathol. **1**:198–216.

53. **Lee J-H, Kim S-H, Han S-H, An J-S, Lee E-S, Kim Y-S**. 2009. Clinicopathological and molecular characteristics of Epstein-Barr virus-associated gastric carcinoma: a meta-analysis. J. Gastroenterol. Hepatol. **24**:354–65.

54. **Murphy G, Pfeiffer R, Camargo MC, Rabkin CS**. 2009. Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location. Gastroenterology **137**:824–33.

55. **Su W-H, Hildesheim A, Chang Y-S**. 2013. Human Leukocyte Antigens and Epstein-Barr Virus-Associated Nasopharyngeal Carcinoma: Old Associations Offer New Clues into the Role of Immunity in Infection-Associated Cancers. Front. Oncol. **3**:299.

56. **Lo KW, Teo PM, Hui AB, To KF, Tsang YS, Chan SY, Mak KF, Lee JC, Huang DP**. 2000. High resolution allelotype of microdissected primary nasopharyngeal carcinoma. Cancer Res. **60**:3348–53.

57. **Chan AS, To KF, Lo KW, Mak KF, Pak W, Chiu B, Tse GM, Ding M, Li X, Lee JC, Huang DP**. 2000. High frequency of chromosome 3p deletion in histologically normal nasopharyngeal epithelia from southern Chinese. Cancer Res. **60**:5365–70.

58. **Chan ASC, To KF, Lo KW, Ding M, Li X, Johnson P, Huang DP**. 2002. Frequent chromosome 9p losses in histologically normal nasopharyngeal epithelia from southern Chinese. Int. J. Cancer **102**:300–3.

59. **Tsang CM, Yip YL, Lo KW, Deng W, To KF, Hau PM, Lau VMY, Takada K, Lui VWY, Lung ML, Chen H, Zeng M, Middeldorp JM, Cheung AL-M, Tsao SW**. 2012. Cyclin D1 overexpression supports stable EBV infection in nasopharyngeal epithelial cells. Proc. Natl. Acad. Sci. U. S. A. **109**:E3473–82.

60. **Tsang CM, Zhang G, Seto E, Takada K, Deng W, Yip YL, Man C, Hau PM, Chen H, Cao Y, Lo KW, Middeldorp JM, Cheung ALM, Tsao SW**. 2010. Epstein-Barr virus infection in immortalized nasopharyngeal epithelial cells: regulation of infection and phenotypic characterization. Int. J. Cancer **127**:1570–83.

61. **Yip YL, Pang PS, Deng W, Tsang CM, Zeng M, Hau PM, Man C, Jin Y, Yuen APW, Tsao SW**. 2013. Efficient immortalization of primary nasopharyngeal epithelial cells for EBV infection study. PLoS One **8**:e78395.

62. **Chan WY, Chan AB, Liu AY, Chow JH, Ng EK, Chung SS**. 2001. Chromosome 11 copy number gains and Epstein-Barr virus-associated malignancies. Diagn. Mol. Pathol. **10**:223–7.

63. **Chan WY, Liu Y, Li CYS, Ng EKW, Chow JHS, Li KKW, Chung SCS**. 2002. Recurrent genomic aberrations in gastric carcinomas associated with Helicobacter pylori and Epstein-Barr virus. Diagn. Mol. Pathol. **11**:127–34.

64. **Zur Hausen A, van Grieken NC, Meijer GA, Hermsen M a. J a., Bloemena E, Meuwissen SGM, Baak JP a., Meijer CJLM, Kuipers EJ, van den Brule AJ**. 2001. Distinct chromosomal aberrations in

Epstein-Barr virus-carrying gastric carcinomas tested by comparative genomic hybridization. Gastroenterology **121**:612–8.

65. **Koriyama C, Akiba S, Minakami Y, Eizuru Y**. 2005. Environmental factors related to Epstein-Barr virus-associated gastric cancer in Japan. J. Exp. Clin. Cancer Res. **24**:547–53.

66. **Klein G, Giovanella BC, Lindahl T, Fialkow PJ, Singh S, Stehlin JS**. 1974. Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. Proc. Natl. Acad. Sci. U. S. A. **71**:4737–41.

67. **Andersson-Anvret M, Forsby N, Klein G, Henle W**. 1977. Relationship between the Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: correlated nucleic acid hybridization and histopathological examination. Int. J. Cancer **20**:486–94.

68. **Old LJ, Boyse EA, Oettgen HF, Harven ED, Geering G, Williamson B, Clifford P**. 1966. Precipitating antibody in human serum to an antigen present in cultured burkitt's lymphoma cells. Proc. Natl. Acad. Sci. U. S. A. **56**:1699–704.

69. **Zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L**. 1970. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature **228**:1056–8.

70. **Raab-Traub N, Flynn K**. 1986. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell **47**:883–9.

71. **Burke AP, Yen TS, Shekitka KM, Sobin LH**. 1990. Lymphoepithelial carcinoma of the stomach with Epstein-Barr virus demonstrated by polymerase chain reaction. Mod. Pathol. **3**:377–80.

72. **Shibata D, Tokunaga M, Uemura Y, Sato E, Tanaka S, Weiss LM**. 1991. Association of Epstein-Barr virus with undifferentiated gastric carcinomas with intense lymphoid infiltration. Lymphoepithelioma-like carcinoma. Am. J. Pathol. **139**:469–74.

73. **Shibata D, Weiss LM**. 1992. Epstein-Barr virus-associated gastric adenocarcinoma. Am. J. Pathol. **140**:769–74.

74. **Tokunaga M, Land CE, Uemura Y, Tokudome T, Tanaka S, Sato E**. 1993. Epstein-Barr virus in gastric carcinoma. Am. J. Pathol. **143**:1250–4.

75. **Yoshizaki T, Kondo S, Wakisaka N, Murono S, Endo K, Sugimoto H, Nakanishi S, Tsuji A, Ito M**. 2013. Pathogenic role of Epstein-Barr virus latent membrane protein-1 in the development of nasopharyngeal carcinoma. Cancer Lett. **337**:1–7.

76. **Takada K**. 2012. Role of EBER and BARF1 in nasopharyngeal carcinoma (NPC) tumorigenesis. Semin. Cancer Biol. **22**:162–5.

77. **Swaminathan S**. 2008. Noncoding RNAs produced by oncogenic human herpesviruses. J. Cell. Physiol. **216**:321–326.

78. **Marquitz AR, Raab-Traub N**. 2012. The role of miRNAs and EBV BARTs in NPC. Semin. Cancer Biol. **22**:166–72.

79. **Lung RW-M, Tong JH-M, To K-F**. 2013. Emerging roles of small Epstein-Barr virus derived non-coding RNAs in epithelial malignancy. Int. J. Mol. Sci. **14**:17378–409.

80. **Lo AK-F, Dawson CW, Jin D-Y, Lo K-W**. 2012. The pathological roles of BART miRNAs in nasopharyngeal carcinoma. J. Pathol. **227**:392–403.

81. **Frappier L**. 2012. Role of EBNA1 in NPC tumourigenesis. Semin. Cancer Biol. **22**:154–61.

82. **Zheng H, Li LL, Hu DS, Deng XY, Cao Y**. 2007. Role of Epstein-Barr virus encoded latent membrane protein 1 in the carcinogenesis of nasopharyngeal carcinoma. Cell. Mol. Immunol. **4**:185–96.

83. **Fukayama M**. 2010. Epstein-Barr virus and gastric carcinoma. Pathol. Int. **60**:337–50.

84. **Young LS, Rickinson AB**. 2004. Epstein-Barr virus: 40 years on. Nat. Rev. Cancer **4**:757–68.

85. **Raab-Traub N**. 2012. Novel mechanisms of EBV-induced oncogenesis. Curr. Opin. Virol. **2**:453–8.

86. **Iwakiri D, Sheen T-S, Chen J-Y, Huang DP, Takada K**. 2005. Epstein-Barr virus-encoded small RNA induces insulin-like growth factor 1 and supports growth of nasopharyngeal carcinoma-derived cell lines. Oncogene **24**:1767–73.

87. **Iwakiri D, Eizuru Y, Tokunaga M, Takada K**. 2003. Autocrine growth of Epstein-Barr virus-positive gastric carcinoma cells mediated by an Epstein-Barr virus-encoded small RNA. Cancer Res. **63**:7062–7.

88. **Samanta M, Iwakiri D, Kanda T, Imaizumi T, Takada K**. 2006. EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. EMBO J. **25**:4207–14.

89. **Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, Imai S, Fujieda M, Kawa K, Takada K**. 2009. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from toll-like receptor 3. J. Exp. Med. **206**:2091-9

90. **Nanbo A, Yoshiyama H, Takada K**. 2005. Epstein-Barr virus-encoded poly(A)- RNA confers resistance to apoptosis mediated through Fas by blocking the PKR pathway in human epithelial intestine 407 cells. J. Virol. **79**:12280–5.

91. **Gilligan K, Sato H, Rajadurai P, Busson P, Young L, Rickinson A, Tursz T, Raab-Traub N**. 1990. Novel transcription from the Epstein-Barr virus terminal EcoRI fragment, DIJhet, in a nasopharyngeal carcinoma. J. Virol. **64**:4948–56.

92. **Smith PR, de Jesus O, Turner D, Hollyoake M, Karstegl CE, Griffin BE, Karran L, Wang Y, Hayward SD, Farrell PJ**. 2000. Structure and coding content of CST (BART) family RNAs of Epstein-Barr virus. J. Virol. **74**:3082–92.

93. **Al-Mozaini M, Bodelon G, Karstegl CE, Jin B, Al-Ahdal M, Farrell PJ**. 2009. Epstein-Barr virus BART gene expression. J. Gen. Virol. **90**:307–16.

94. **Gilligan KJ, Rajadurai P, Lin JC, Busson P, Abdel-Hamid M, Prasad U, Tursz T, Raab-Traub N**. 1991. Expression of the Epstein-Barr virus BamHI A fragment in nasopharyngeal carcinoma: evidence for a viral protein expressed in vivo. J. Virol. **65**:6252–9.

95. **Seto E, Yang L, Middeldorp J, Sheen T-S, Chen J-Y, Fukayama M, Eizuru Y, Ooka T, Takada K**. 2005. Epstein-Barr virus (EBV)-encoded BARF1 gene is expressed in nasopharyngeal carcinoma and EBV-associated gastric carcinoma tissues in the absence of lytic gene expression. J. Med. Virol. **76**:82–8.

96. **Decaussin G, Sbih-Lammali F, de Turenne-Tessier M, Bouguermouh A, Ooka T**. 2000. Expression of BARF1 gene encoded by Epstein-Barr virus in nasopharyngeal carcinoma biopsies. Cancer Res. **60**:5584–8.

97. **Zhang CX, Decaussin G, Daillie J, Ooka T**. 1988. Altered expression of two Epstein-Barr virus early genes localized in BamHI-A in nonproducer Raji cells. J. Virol. **62**:1862–9.

98. **Qiu J, Cosmopoulos K, Pegtel M, Hopmans E, Murray P, Middeldorp J, Shapiro M, Thorley-Lawson D a**. 2011. A novel persistence associated EBV miRNA expression profile is disrupted in neoplasia. PLoS Pathog. **7**:e1002193.

99. **Bartel DP**. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116**:281–97.

100. **Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson D a**. 2009. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. PLoS Pathog. **5**:e1000496.

101. **Perera RAPM, Samaranayake LP, Tsang CSP**. 2010. Shedding dynamics of Epstein-Barr virus: A type 1 carcinogen. Arch. Oral Biol. **55**:639–47.

102. **Barth S, Pfuhl T, Mamiani A, Ehses C, Roemer K, Kremmer E, Jäker C, Höck J, Meister G, Grässer F a**. 2008. Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. Nucleic Acids Res. **36**:666–75.

103. **Riley KJ, Rabinowitz GS, Yario T a, Luna JM, Darnell RB, Steitz J a**. 2012. EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J. **31**:2207–21.

104. **Ramakrishnan R, Donahue H, Garcia D, Tan J, Shimizu N, Rice AP, Ling PD**. 2011. Epstein-Barr virus BART9 miRNA modulates LMP1 levels and affects growth rate of nasal NK T cell lymphomas. PLoS One **6**:e27271.

105. **Lo AKF, To KF, Lo KW, Lung RWM, Hui JWY, Liao G, Hayward SD**. 2007. Modulation of LMP1 protein expression by EBV-encoded microRNAs. Proc. Natl. Acad. Sci. U. S. A. **104**:16164–9.

106. **Lung RW-M, Tong JH-M, Sung Y-M, Leung P-S, Ng DC-H, Chau S-L, Chan AW-H, Ng EK-O, Lo K-W, To K-F**. 2009. Modulation of LMP2A expression by a newly identified Epstein-Barr virus-encoded microRNA miR-BART22. Neoplasia **11**:1174–84.

107. **Yates JL, Warren N, Sugden B**. 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature **313**:812–5.

108. **Yates J, Warren N, Reisman D, Sugden B**. 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. U. S. A. **81**:3806–10.

109. **Rawlins DR, Milman G, Hayward SD, Hayward GS**. 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42**:859–68.

110. **Yates JL, Guan N**. 1991. Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. J. Virol. **65**:483–8.

111. **Gahn TA, Sugden B**. 1995. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. J. Virol. **69**:2633–6.

112. **Cheng T-C, Hsieh S-S, Hsu W-L, Chen Y-F, Ho H-H, Sheu L-F**. 2010. Expression of Epstein-Barr nuclear antigen 1 in gastric carcinoma cells is associated with enhanced tumorigenicity and reduced cisplatin sensitivity. Int. J. Oncol. **36**:151–60.

113. **Sheu LF, Chen A, Meng CL, Ho KC, Lee WH, Leu FJ, Chao CF**. 1996. Enhanced malignant progression of nasopharyngeal carcinoma cells mediated by the expression of Epstein-Barr nuclear antigen 1 in vivo. J. Pathol. **180**:243–8.

114. **Sivachandran N, Dawson CW, Young LS, Liu F-F, Middeldorp J, Frappier L**. 2012. Contributions of the Epstein-Barr virus EBNA1 protein to gastric carcinoma. J. Virol. **86**:60–8.

115. **Sivachandran N, Sarkari F, Frappier L**. 2008. Epstein-Barr nuclear antigen 1 contributes to nasopharyngeal carcinoma through disruption of PML nuclear bodies. PLoS Pathog. **4**:e1000170.

116. **Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM, Klein G, Kurilla MG, Masucci MG**. 1995. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. Nature **375**:685–8.

117. **Brooks L, Yao QY, Rickinson AB, Young LS**. 1992. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J. Virol. **66**:2689–97.

118. **Tsao SW, Tramoutanis G, Dawson CW, Lo AKF, Huang DP**. 2002. The significance of LMP1 expression in nasopharyngeal carcinoma. Semin. Cancer Biol. **12**:473–87.

119. **Liu Y, Wang X, Lo AKF, Wong YC, Cheung ALM, Tsao SW**. 2002. Latent membrane protein-1 of Epstein-Barr virus inhibits cell growth and induces sensitivity to cisplatin in nasopharyngeal carcinoma cells. J. Med. Virol. **66**:63–9.

120. **Lu JJ, Chen JY, Hsu TY, Yu WC, Su IJ, Yang CS**. 1996. Induction of apoptosis in epithelial cells by Epstein-Barr virus latent membrane protein 1. J. Gen. Virol. **77 (Pt 8)**:1883–92.

121. **Lee DY, Sugden B**. 2008. The latent membrane protein 1 oncogene modifies B-cell physiology by regulating autophagy. Oncogene **27**:2833–42.

122. **Miller WE, Cheshire JL, Raab-Traub N**. 1998. Interaction of tumor necrosis factor receptor-associated factor signaling proteins with the latent membrane protein 1 PXQXT motif is essential for induction of epidermal growth factor receptor expression. Mol. Cell. Biol. **18**:2835–44.

123. **Kung C-P, Meckes DG, Raab-Traub N**. 2011. Epstein-Barr virus LMP1 activates EGFR, STAT3, and ERK through effects on PKCdelta. J. Virol. **85**:4399–408.

124. **Terrin L, Dal Col J, Rampazzo E, Zancai P, Pedrotti M, Ammirabile G, Bergamin S, Rizzo S, Dolcetti R, De Rossi A**. 2008. Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. J. Virol. **82**:10175–87.

125. **Hanahan D, Weinberg R a**. 2011. Hallmarks of cancer: the next generation. Cell **144**:646–74.

126. **Hanahan D, Weinberg RA**. 2000. The hallmarks of cancer. Cell **100**:57–70.

127. **Crowe DL, Nguyen DC, Ohannessian A**. 2005. Mechanism of telomerase repression during terminal differentiation of normal epithelial cells and squamous carcinoma lines. Int. J. Oncol. **27**:847–54.

128. **Feller L, Altini M, Khammissa R a G, Chandran R, Bouckaert M, Lemmer J**. 2013. Oral mucosal immunity. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. **116**:576–83.

129. **Kumar SKS, Zain RB, Ismail SM, Cheong SC**. 2005. Human telomerase reverse transcriptase expression in oral carcinogenesis--a preliminary report. J. Exp. Clin. Cancer Res. **24**:639–46.

130. **O'Flatharta C, Leader M, Kay E, Flint SR, Toner M, Robertson W, Mabruk MJEMF**. 2002. Telomerase activity detected in oral lichen planus by RNA in situ hybridisation: not a marker for malignant transformation. J. Clin. Pathol. **55**:602–7.

131. **Yasumoto S, Kunimura C, Kikuchi K, Tahara H, Ohji H, Yamamoto H, Ide T, Utakoji T**. 1996. Telomerase activity in normal human epithelial cells. Oncogene **13**:433–9.

132. **Patel V, Iglesias-Bartolome R, Siegele B, Marsh CA, Leelahavanichkul K, Molinolo AA, Gutkind JS**. 2011. Cellular systems for studying human oral squamous cell carcinomas. Adv. Exp. Med. Biol. **720**:27–38.

133. **Rovedo M, Longnecker R**. 2007. Epstein-barr virus latent membrane protein 2B (LMP2B) modulates LMP2A activity. J. Virol. **81**:84–94.

134. **Miller CL, Burkhardt a L, Lee JH, Stealey B, Longnecker R, Bolen JB, Kieff E**. 1995. Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. Immunity **2**:155–66.

135. **Miller CL, Lee JH, Kieff E, Longnecker R**. 1994. An integral membrane protein (LMP2) blocks reactivation of Epstein-Barr virus from latency following surface immunoglobulin crosslinking. Proc. Natl. Acad. Sci. U. S. A. **91**:772–6.

136. **Caldwell RG, Wilson JB, Anderson SJ, Longnecker R**. 1998. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity **9**:405–11.

137. **Longan L, Longnecker R**. 2000. Epstein-Barr virus latent membrane protein 2A has no growth-altering effects when expressed in differentiating epithelia. J. Gen. Virol. **81**:2245–52.

138. **Fukuda M, Longnecker R**. 2007. Epstein-Barr virus latent membrane protein 2A mediates transformation through constitutive activation of the Ras/PI3-K/Akt Pathway. J. Virol. **81**:9299–306.

139. **Moody CA, Scott RS, Amirghahari N, Nathan C, Young LS, Dawson CW, Sixbey JW**. 2005. Modulation of the cell growth regulator mTOR by Epstein-Barr virus-encoded LMP2A. J. Virol. **79**:5499–506.

140. **Moody CA, Scott RS, Su T, Sixbey JW**. 2003. Length of Epstein-Barr virus termini as a determinant of epithelial cell clonal emergence. J. Virol. **77**:8555–61.

141. **Scholle F, Bendt KM, Raab-Traub N**. 2000. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J. Virol. **74**:10681–9.

142. **Lu J, Lin W-H, Chen S-Y, Longnecker R, Tsai S-C, Chen C-L, Tsai C-H**. 2006. Syk tyrosine kinase mediates Epstein-Barr virus latent membrane protein 2A-induced cell migration in epithelial cells. J. Biol. Chem. **281**:8806–14.

143. **Hutt-Fletcher LM**. 2007. Epstein-Barr virus entry. J. Virol. **81**:7825–32.

144. **Connolly S a, Jackson JO, Jardetzky TS, Longnecker R**. 2011. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat. Rev. Microbiol. **9**:369–81.

145. **Ogembo JG, Kannan L, Ghiran I, Nicholson-Weller A, Finberg RW, Tsokos GC, Fingeroth JD**. 2013. Human Complement Receptor Type 1/CD35 Is an Epstein-Barr Virus Receptor. Cell Rep. **3**:371–385.

146. **Fingeroth JD, Diamond ME, Sage DR, Hayman J, Yates JL**. 1999. CD21-Dependent infection of an epithelial cell line, 293, by Epstein-Barr virus. J. Virol. **73**:2115–25.

147. **Sixbey JW, Yao QY**. 1992. Immunoglobulin A-induced shift of Epstein-Barr virus tissue tropism. Science **255**:1578–80.

148. **Gan YJ, Chodosh J, Morgan A, Sixbey JW**. 1997. Epithelial cell polarization is a determinant in the infectious outcome of immunoglobulin A-mediated entry by Epstein-Barr virus. J. Virol. **71**:519–26.

149. **Molesworth SJ, Lake CM, Borza CM, Turk SM, Hutt-Fletcher LM**. 2000. Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. J. Virol. **74**:6324–32.

150. **Oda T, Imai S, Chiba S, Takada K**. 2000. Epstein-Barr virus lacking glycoprotein gp85 cannot infect B cells and epithelial cells. Virology **276**:52–8.

151. **Stewart PL, Nemerow GR**. 2007. Cell integrins: commonly used receptors for diverse viral pathogens. Trends Microbiol. **15**:500–7.

152. **Jolly CL, Sattentau QJ**. 2013. Attachment factors. Adv. Exp. Med. Biol. **790**:1–23.

153. **Tugizov SM, Berline JW, Palefsky JM**. 2003. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. Nat. Med. **9**:307–314.

154. **Xiao J, Palefsky JM, Herrera R, Tugizov SM**. 2007. Characterization of the Epstein-Barr virus glycoprotein BMRF-2. Virology **359**:382–396.

155. **Xiao J, Palefsky JM, Herrera R, Berline JW, Tugizov SM**. 2008. The Epstein-Barr virus BMRF-2 protein facilitates virus attachment to oral epithelial cells. Virology **370**:430–442.

156. **Xiao J, Palefsky JM, Herrera R, Berline J, Tugizov SM**. 2009. EBV BMRF-2 facilitates cell-to-cell spread of virus within polarized oral epithelial cells. Virology **388**:335–43.

157. **Hutt-Fletcher L, Chesnokova L**. 2010. Integrins as triggers of Epstein-Barr virus fusion and epithelial cell infection. Virulence **106**:20464–9.

158. **Chesnokova LS, Hutt-Fletcher LM**. 2011. Fusion of Epstein-Barr Virus with Epithelial Cells Can Be Triggered by αvβ5 in Addition to αvβ6 and αvβ8, and Integrin Binding Triggers a Conformational Change in Glycoproteins gHgL. J. Virol. **85**:13214–23.

159. **Chesnokova LS, Nishimura SL, Hutt-Fletcher LM**. 2009. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins alphavbeta6 or alphavbeta8. Proc. Natl. Acad. Sci. U. S. A. **106**:20464–9.

160. **Tugizov SM, Herrera R, Palefsky JM**. 2013. Epstein-Barr virus transcytosis through polarized oral epithelial cells. J. Virol. **87**:8179–94.

161. **Wang X, Kenyon WJ, Li Q, Müllberg J, Hutt-Fletcher LM**. 1998. Epstein-Barr virus uses different complexes of glycoproteins gH and gL to infect B lymphocytes and epithelial cells. J. Virol. **72**:5552–8.

162. **Imai S, Nishikawa J, Takada K**. 1998. Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells. J. Virol. **72**:4371–8.

163. **Shannon-Lowe CD, Neuhierl B, Baldwin G, Rickinson AB, Delecluse H-J**. 2006. Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. Proc. Natl. Acad. Sci. U. S. A. **103**:7065–70.

164. **Feederle R, Neuhierl B, Bannert H, Geletneky K, Shannon-Lowe C, Delecluse H-J**. 2007. Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection. Int. J. Cancer **121**:588–94.

165. **Tsai M-H, Raykova A, Klinke O, Bernhardt K, Gärtner K, Leung CS, Geletneky K, Sertel S, Münz C, Feederle R, Delecluse H-J**. 2013. Spontaneous Lytic Replication and Epitheliotropism Define an Epstein-Barr Virus Strain Found in Carcinomas. Cell Rep. 1–13.

166. **Turk SM, Jiang R, Chesnokova LS, Hutt-Fletcher LM**. 2006. Antibodies to gp350/220 enhance the ability of Epstein-Barr virus to infect epithelial cells. J. Virol. **80**:9628–33.

167. **Shannon-Lowe C, Rowe M**. 2011. Epstein-Barr Virus Infection of Polarized Epithelial Cells via the Basolateral Surface by Memory B Cell-Mediated Transfer Infection. PLoS Pathog. **7**:e1001338.

168. **Otczyk DC, Cripps AW**. 2010. Mucosal immunization: A realistic alternative. Hum. Vaccin. **6**:978–1006.

169. **Nave H, Gebert a, Pabst R**. 2001. Morphology and immunology of the human palatine tonsil. Anat. Embryol. (Berl). **204**:367–73.

170. **Ogasawara N, Kojima T, Go M, Takano K, Kamekura R, Ohkuni T, Koizumi J, Masaki T, Fuchimoto J, Obata K, Kurose M, Shintani T, Sawada N, Himi T**. 2011. Epithelial barrier and antigen uptake in lymphoepithelium of human adenoids. Acta Otolaryngol. **131**:116–23.

171. **Squier CA, Kremer MJ**. 2001. Biology of oral mucosa and esophagus. J. Natl. Cancer Inst. Monogr. **52242**:7–15.

172. **Jones KB, Klein OD**. 2013. Oral epithelial stem cells in tissue maintenance and disease: the first steps in a long journey. Int. J. Oral Sci. **5**:121–9.

173. **Feller LL, Khammissa RR, Kramer BB, Lemmer JJ**. 2013. Oral squamous cell carcinoma in relation to field precancerisation: pathobiology. Cancer Cell Int. **13**:31.

174. **Nandakumar J, Cech TR**. 2013. Finding the end: recruitment of telomerase to telomeres. Nat. Rev. Mol. Cell Biol. **14**:69–82.

175. **Majerská J, Sýkorová E, Fajkus J**. 2011. Non-telomeric activities of telomerase. Mol. Biosyst. **7**:1013–23.

176. **Ding D, Zhou J, Wang M, Cong Y-S**. 2013. Implications of telomere-independent activities of telomerase reverse transcriptase in human cancer. FEBS J. **280**:3205–11.

177. **Chiodi I, Mondello C**. 2012. Telomere-independent functions of telomerase in nuclei, cytoplasm, and mitochondria. Front. Oncol. **2**:133.

178. **Ghosh A, Saginc G, Leow SC, Khattar E, Shin EM, Yan TD, Wong M, Zhang Z, Li G, Sung W-K, Zhou J, Chng WJ, Li S, Liu E, Tergaonkar V**. 2012. Telomerase directly regulates NF-κB-dependent transcription. Nat. Cell Biol. **14**:1270–81.

179. **Park J-I, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, Chang W, Meng Z, Cheung P, Ji H, McLaughlin M, Veenstra TD, Nusse R, McCrea PD, Artandi SE**. 2009. Telomerase modulates Wnt signalling by association with target gene chromatin. Nature **460**:66–72.

180. **Choi J, Southworth LK, Sarin KY, Venteicher AS, Ma W, Chang W, Cheung P, Jun S, Artandi MK, Shah N, Kim SK, Artandi SE**. 2008. TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. PLoS Genet. **4**:e10.

181. **Herrmann K, Frangou P, Middeldorp J, Niedobitek G**. 2002. Epstein-Barr virus replication in tongue epithelial cells. J. Gen. Virol. **83**:2995–8.

182. **Frangou P, Buettner M, Niedobitek G**. 2005. Epstein-Barr virus (EBV) infection in epithelial cells in vivo: rare detection of EBV replication in tongue mucosa but not in salivary glands. J. Infect. Dis. **191**:238–42.

183. **Buettner M, Lang A, Tudor CS, Meyer B, Cruchley A, Barros MHM, Farrell PJ, Jäck H-M, Schuh W, Niedobitek G**. 2012. Lytic Epstein-Barr virus infection in epithelial cells but not in B-lymphocytes is dependent on Blimp1. J. Gen. Virol. **93**:1059–64.

184. **Laichalk LL, Thorley-Lawson DA**. 2005. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. J. Virol. **79**:1296–307.

185. **Yip Y-L, Tsang C-M, Deng W, Cheung P-Y, Jin Y, Cheung AL-M, Lung ML, Tsao S-W**. 2010. Expression of Epstein-Barr virus-encoded LMP1 and hTERT extends the life span and immortalizes primary cultures of nasopharyngeal epithelial cells. J. Med. Virol. **82**:1711–23.

186. **Wille CK, Nawandar DM, Panfil AR, Ko MM, Hagemeier SR, Kenney SC**. 2013. Viral genome methylation differentially affects the ability of BZLF1 versus BRLF1 to activate Epstein-Barr virus lytic gene expression and viral replication. J. Virol. **87**:935–50.

187. **Giunco S, Dolcetti R, Keppel S, Celeghin A, Indraccolo S, Dal Col J, Mastorci K, De Rossi A**. 2013. hTERT inhibition triggers Epstein-Barr virus lytic cycle and apoptosis in immortalized and transformed B cells: a basis for new therapies. Clin. Cancer Res. **19**:2036–47.

188. **Terrin L, Dolcetti R, Corradini I, Indraccolo S, Dal Col J, Bertorelle R, Bonaldi L, Esposito G, De Rossi A**. 2007. hTERT inhibits the Epstein-Barr virus lytic cycle and promotes the proliferation of primary B lymphocytes: implications for EBV-driven lymphomagenesis. Int. J. Cancer **121**:576–87.

189. **Waterer GW**. 2012. Airway defense mechanisms. Clin. Chest Med. **33**:199–209.

190. **Diamond G, Beckloff N, Ryan LK**. 2008. Host defense peptides in the oral cavity and the lung: similarities and differences. J. Dent. Res. **87**:915–27.

191. **Ryu J-H, Kim C-H, Yoon J-H**. 2010. Innate immune responses of the airway epithelium. Mol. Cells **30**:173–83.

192. **Vareille M, Kieninger E, Edwards MR, Regamey N**. 2011. The airway epithelium: soldier in the fight against respiratory viruses. Clin. Microbiol. Rev. **24**:210–29.

193. **Vroling a B, Fokkens WJ, van Drunen CM**. 2008. How epithelial cells detect danger: aiding the immune response. Allergy **63**:1110–23.

194. **Hashimoto C, Hudson KL, Anderson K V**. 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell **52**:269–79.

195. **Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann J a**. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86**:973–83.

196. **Medzhitov R, Preston-Hurlburt P, Janeway CA**. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature **388**:394–7.

197. **Kawai T, Akira S**. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. **11**:373–84.

198. **Kumar H, Kawai T, Akira S**. 2009. Toll-like receptors and innate immunity. Biochem. Biophys. Res. Commun. **388**:621–5.

199. **Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S**. 2008. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe **3**:352–63.

200. **Cai M, Li M, Zheng C**. 2012. Herpesviral infection and Toll-like receptor 2. Protein Cell **3**:590–601.

201. **Paludan SR, Bowie AG, Horan K a, Fitzgerald K a**. 2011. Recognition of herpesviruses by the innate immune system. Nat. Rev. Immunol. **11**:143–54.

202. **West J a, Gregory SM, Damania B**. 2012. Toll-like receptor sensing of human herpesvirus infection. Front. Cell. Infect. Microbiol. **2**:122.

203. **Ariza M-E, Glaser R, Kaumaya PTP, Jones C, Williams M V**. 2009. The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. J. Immunol. **182**:851–9.

204. **Fiola S, Gosselin D, Takada K, Gosselin J**. 2010. TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells. J. Immunol. **185**:3620–31.

205. **Gaudreault E, Fiola S, Olivier M, Gosselin J**. 2007. Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2. J. Virol. **81**:8016–24.

206. **Martin HJ, Lee JM, Walls D, Hayward SD**. 2007. Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. J. Virol. **81**:9748–58.

207. **Vérillaud B, Gressette M, Morel Y, Paturel C, Herman P, Lo KW, Tsao SW, Wassef M, Jimenez-Pailhes A-S, Busson P**. 2012. Toll-like receptor 3 in Epstein-Barr virus-associated nasopharyngeal carcinomas: consistent expression and cytotoxic effects of its synthetic ligand poly(A:U) combined to a Smac-mimetic. Infect. Agent. Cancer **7**:36.

208. **Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A**. 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat. Med. **10**:871–5.

209. **Younesi V, Shirazi FG, Memarian A, Amanzadeh A, Jeddi-Tehrani M, Shokri F**. 2014. Assessment of the effect of TLR7/8, TLR9 agonists and CD40 ligand on the transformation efficiency of Epstein-Barr virus in human B lymphocytes by limiting dilution assay. Cytotechnology **66**:95–105.

210. **Iskra S, Kalla M, Delecluse H-J, Hammerschmidt W, Moosmann A**. 2010. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. J. Virol. **84**:3612–23.

211. **Ladell K, Dorner M, Zauner L, Berger C, Zucol F, Bernasconi M, Niggli FK, Speck RF, Nadal D**. 2007. Immune activation suppresses initiation of lytic Epstein-Barr virus infection. Cell. Microbiol. **9**:2055–69.

212. **Zauner L, Melroe GT, Sigrist J a, Rechsteiner MP, Dorner M, Arnold M, Berger C, Bernasconi M, Schaefer BW, Speck RF, Nadal D**. 2010. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. Oncogene **29**:4588–98.

213. **Ueda S, Uchiyama S, Azzi T, Gysin C, Berger C, Bernasconi M, Harabuchi Y, Zinkernagel AS, Nadal D**. 2014. Oropharyngeal group a streptococcal colonization disrupts latent epstein-barr virus infection. J. Infect. Dis. **209**:255–64.

214. **Siennicka J, Trzcińska A, Cześcik A, Dunal-Szczepaniak M, Lagosz B**. 2013. The influence of toll-like receptor stimulation on expression of EBV lytic genes. Pol. J. Microbiol. **62**:237–42.

215. **Claeys S, de Belder T, Holtappels G, Gevaert P, Verhasselt B, van Cauwenberge P, Bachert C**. 2003. Human beta-defensins and toll-like receptors in the upper airway. Allergy **58**:748–53.

216. **Hornef MW, Bogdan C**. 2005. The role of epithelial Toll-like receptor expression in host defense and microbial tolerance. J. Endotoxin Res. **11**:124–8.

217. **Lange MJ, Lasiter JC, Misfeldt ML**. 2009. Toll-like receptors in tonsillar epithelial cells. Int. J. Pediatr. Otorhinolaryngol. **73**:613–21.

218. **Swaminathan V, Prakasam S, Puri V, Srinivasan M**. 2013. Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis. J. Periodontal Res. **48**:757–65.

219. **Vandermeer J, Sha Q, Lane AP, Schleimer RP**. 2004. Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and toll-like receptors. Arch. Otolaryngol. Head. Neck Surg. **130**:1374–80.

220. **Sha Q, Truong-Tran AQ, Plitt JR, Beck L a, Schleimer RP**. 2004. Activation of airway epithelial cells by toll-like receptor agonists. Am. J. Respir. Cell Mol. Biol. **31**:358–64.

221. **Platz J, Beisswenger C, Dalpke A, Koczulla R, Pinkenburg O, Vogelmeier C, Bals R**. 2004. Microbial DNA induces a host defense reaction of human respiratory epithelial cells. J. Immunol. **173**:1219–23.

222. **Mempel M, Voelcker V, Köllisch G, Plank C, Rad R, Gerhard M, Schnopp C, Fraunberger P, Walli AK, Ring J, Abeck D, Ollert M**. 2003. Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by Staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. J. Invest. Dermatol. **121**:1389–96.

223. **Gilcrease MZ**. 2007. Integrin signaling in epithelial cells. Cancer Lett. **247**:1–25.

224. **Watt FM**. 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J. **21**:3919–26.

225. **Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JH, Meyerson M, Weinberg RA**. 1999. Inhibition of telomerase limits the growth of human cancer cells. Nat. Med. **5**:1164–70.

226. **Peterson WD, Stulberg CS, Simpson WF**. 1971. A permanent heteroploid human cell line with type B glucose-6-phosphate dehydrogenase. Proc. Soc. Exp. Biol. Med. **136**:1187–91.

227. **Peterson WD, Stulberg CS, Swanborg NK, Robinson AR**. 1968. Glucose-6-phosphate dehydrogenase isoenzymes in human cell cultures determined by sucrose-agar gel and cellulose acetate zymograms. Proc. Soc. Exp. Biol. Med. **128**:772–6.

228. **Gey GO, Coffman WD, Kubicek MT**. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. **12**:264–5.

229. **Takimoto T, Furukawa M, Hatano M, Umeda R**. 1984. Epstein-Barr virus nuclear antigen-positive nasopharyngeal hybrid cells. Ann. Otol. Rhinol. Laryngol. **93**:166–9.

230. **Niederman JC**. 1982. Infectious mononucleosis: observations on transmission. Yale J. Biol. Med. **55**:259–64.

231. **Niederman JC, Miller G, Pearson HA, Pagano JS, Dowaliby JM**. 1976. Infectious mononucleosis. Epstein-Barr-virus shedding in saliva and the oropharynx. N. Engl. J. Med. **294**:1355–9.

232. **Miller G, Niederman JC, Andrews LL**. 1973. Prolonged oropharyngeal excretion of Epstein-Barr virus after infectious mononucleosis. N. Engl. J. Med. **288**:229–32.

233. **Strauch B, Andrews LL, Siegel N, Miller G**. 1974. Oropharyngeal excretion of Epstein-Barr virus by renal transplant recipients and other patients treated with immunosuppressive drugs. Lancet **1**:234–7.

234. **Morgan DG, Niederman JC, Miller G, Smith HW, Dowaliby JM**. 1979. Site of Epstein-Barr virus replication in the oropharynx. Lancet **2**:1154–7.

235. **Niedobitek G, Young LS, Lau R, Brooks L, Greenspan D, Greenspan JS, Rickinson a B**. 1991. Epstein-Barr virus infection in oral hairy leukoplakia: virus replication in the absence of a detectable latent phase. J. Gen. Virol. **72 (Pt 12**:3035–46.

236. **Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, Petersen V, Freese UK**. 1985. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. N. Engl. J. Med. **313**:1564–71.

237. **Walling DM, Flaitz CM, Nichols CM, Hudnall SD, Adler-Storthz K**. 2001. Persistent productive Epstein-Barr virus replication in normal epithelial cells in vivo. J. Infect. Dis. **184**:1499–507.

238. **Walling DM, Flaitz CM, Nichols CM**. 2003. Epstein-Barr virus replication in oral hairy leukoplakia: response, persistence, and resistance to treatment with valacyclovir. J. Infect. Dis. **188**:883–90.

239. **Lau R, Middeldorp J, Farrell PJ**. 1993. Epstein-Barr virus gene expression in oral hairy leukoplakia. Virology **195**:463–74.

240. **Pegtel D, Middeldorp JM**. 2004. Epstein-Barr virus infection in ex vivo tonsil epithelial cell cultures of asymptomatic carriers. J. Virol. **78**:12613–12624.

241. **Niedobitek G, Agathanggelou A, Herbst H, Whitehead L, Wright DH, Young LS**. 1997. Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells. J. Pathol. **182**:151–9.

242. **Niedobitek G, Agathanggelou A, Steven N, Young LS**. 2000. Epstein-Barr virus (EBV) in infectious mononucleosis: detection of the virus in tonsillar B lymphocytes but not in desquamated oropharyngeal epithelial cells. Mol. Pathol. **53**:37–42.

243. **Hoover SE, Kawada J, Wilson W, Cohen JI**. 2008. Oropharyngeal shedding of Epstein-Barr virus in the absence of circulating B cells. J. Infect. Dis. **198**:318–23.

244. **Maloney DG, Liles TM, Czerwinski DK, Waldichuk C, Rosenberg J, Grillo-Lopez A, Levy R**. 1994. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. Blood **84**:2457–66.

245. **Genberg H, Hansson A, Wernerson A, Wennberg L, Tydén G**. 2006. Pharmacodynamics of rituximab in kidney allotransplantation. Am. J. Transplant **6**:2418–28.

246. **Jiang R, Scott RS, Hutt-Fletcher LM**. 2006. Epstein-Barr virus shed in saliva is high in B-cell-tropic glycoprotein gp42. J. Virol. **80**:7281–3.

IX ATTACHMENTS

IX.1 Manuscript I: Telomerase activity enhances Epstein-Barr virus gene expression and contributes to virus maintenance in epithelial cells

Jürgen Rac[1,2], Florian Haas[1,2], Andrina Schumacher[1,2], Michele Bernasconi[1,2], Roberto F. Speck[3], David Nadal[1,2,*]

[1]Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital of Zurich, University of Zurich, Zurich, Switzerland

[2]Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland

[3]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, Zurich, Switzerland

*Corresponding author:
David Nadal, M.D.
University Children's Hospital of Zurich
Division of Infectious Diseases and Hospital Epidemiology
Steinwiesstrasse 75
CH-8032 Zurich
Switzerland
E-Mail: david.nadal@kispi.uzh.ch
Phone +41 44 266 7562;
FAX +41 44 266 8072

Manuscript in preparation

Authors' contributions: J.R. planned, performed and analyzed experiments shown in Figure 1-4 and wrote the manuscript. A.S. generated the cell line AGS-hT. Remaining authors supervised and supported the study design and revised the Manuscript.

IX.1.1 Abstract

Epstein-Barr virus (EBV) is a ubiquitous virus, tightly associated with various lymphoid and epithelial malignancies. Epithelial stem cells and transiently proliferating cells within the basal layers of epithelial tissues exhibit unlimited self-renewal capacity mediated by activated telomerase activity. During cell division, these cells might acquire genetic and epigenetic alterations generating an environment to support persistent EBV infection. Amongst these alterations, loss of p16 and overexpression of cyclin D1 appear to be crucial for the establishment of an EBV infection in epithelial cells and are known to be common features in EBV associated epithelial tumors. However, the impact of telomerase activity on the infection of epithelial cells remains to be elucidated. Therefore, we generated epithelial model cell lines with increased telomerase activity by stable ectopic expression of hTERT, the rate-limiting component of the telomerase complex. Conversely, we suppressed telomerase activity by stable expression of a catalytically inactive, dominant negative hTERT mutant. We then performed infection experiments using a recombinant wild type EBV strain encoding GFP and determined infection rates by flow cytometry as well as EBV gene expression by RT-qPCR. We found that the infection is strongly depending on the cellular context and that infection frequencies partly depend on telomerase activity. Moreover, epithelial cells with increased telomerase activity showed up-regulation mainly of latent EBV genes as compared to their corresponding control cell lines. We conclude that increased telomerase activity directly acts on the EBV infection of epithelial cells by facilitating latent EBV gene expression and therefore contributing to EBV maintenance in epithelial cells.

IX.1.2 Introduction

The Epstein-Barr virus (EBV), a member of the γ-herpesvirus family, is very successful in infecting over 90% of the adult human population (Rickinson & Kieff, 2007). Primary infection with EBV is usually asymptomatic or causes unspecific symptoms. In individuals older than 5 years of age, however, primary infection may manifest as infectious mononucleosis. Importantly, EBV has been linked to various lymphoid and epithelial cell malignancies, e.g. Burkitt's lymphoma (BL), Hodgkin's lymphoma, gastric carcinoma (GC) and nasopharyngeal carcinoma (NPC) (Kieff & Rickinson, 2007). Upon infection of its main target cells, the B-cells, where EBV eventually persists, the virus enters its default mode of infection called latency (Rickinson & Kieff, 2007). This goes along with restricted viral gene expression and therefore a maximum level of immune evasion (Griffin *et al.*, 2010; Hislop *et*

al., 2007; Rickinson & Kieff, 2007; Speck & Ganem, 2010; Taylor & Blackbourn, 2011). By contrast, the lytic replication mode or so called productive infection results in production of infectious progeny virus (Kieff & Rickinson, 2007).

As an orally transmitted microbe, EBV has to overcome the oral mucosal epithelium to be shed into saliva and spread to susceptible hosts. Thus, the oral mucosal epithelium plays a crucial role in EBV life cycle. Since EBV was the first human oncogenic virus discovered (Epstein *et al.*, 1964) it is not surprising that there are tight associations with epithelial cell carcinomas apart from B-cell derived tumors like BL. It is suggested that in NPC and in about 10% of GCs EBV exhibits an important role in tumor development and progression. Indeed, all EBV positive tumor cells appear to be a result of monoclonal outgrowth of one single infected cell (Gu *et al.*, 2012; Iizasa *et al.*, 2012; Li *et al.*, 2006; Lo *et al.*, 2004, 2012; Rickinson & Kieff, 2007; Takada, 2012). Nevertheless, EBV by itself does not transform epithelial cells *in vitro.* Interestingly, low-grade dysplastic precursor lesions in both NPC and GC have been shown to be EBV negative (Chan *et al.*, 2000, 2002; Zur Hausen *et al.*, 2004). This lead to a model where preexisting cellular alterations generate cells that are either more susceptible to EBV infection or are able to support a persistent latent EBV infection, contributing to tumor progression and malignant transformation (Gu *et al.*, 2012; Iizasa *et al.*, 2012; Lo *et al.*, 2004, 2012; Rickinson & Kieff, 2007; Tsao *et al.*, 2012; Yoshizaki *et al.*, 2013).

The oral mucosal epithelium is a dynamic tissue with a distinct multilayer architecture (Patel *et al.*, 2011). Its basement membrane separates the epithelium from the underlying *lamina propria* and ensures correct and directed migration and differentiation of the overlying epithelial cells towards the surface of the epithelium. The *stratum basale*, a single layer of cells resting on the basement membrane, is most important for tissue hemostasis. The *stratum basale* harbors a small sub-population of epithelial stem cells, which can undergo mitotic division and give rise to transiently proliferating progenitor cells (Feller *et al.*, 2013; Patel *et al.*, 2011). The transiently proliferating cells then can generate daughter cells that migrate and differentiate through the *stratum spinosum* and *stratum granulosum* towards the epithelial surface, the *stratum corneum*. Epithelial stem cells are known to have an increased expression and activity of the human telomerase reverse transcriptase (hTERT), the rate-limiting component of the telomerase complex, to ensure indefinite proliferation and continuous self-renewal capacity (Crowe *et al.*, 2005; Feller *et al.*, 2013; Kumar *et al.*, 2005; O'Flatharta *et al.*, 2002; Yasumoto *et al.*, 1996). Due to their self-renewal capacity, these stem and

transiently proliferating cells might accumulate genetic and epigenetic alterations (Feller *et al.*, 2013; Patel *et al.*, 2011) and therefore acquire a precancerous phenotype.

EBV is rapidly lost from infected primary epithelial cells or from epithelial tumor cells *in vitro* (Glaser *et al.*, 1989; Shannon-Lowe *et al.*, 2009; Wu *et al.*, 2003; Yao *et al.*, 1990). Nonetheless, it was possible to establish hTERT-immortalized nasopharyngeal epithelial (NPE) cell clones, able to support a long-term infection by EBV. It appeared that loss of the tumor suppressor p16, or its inactivation, and cyclin D1 overexpression are crucial for the establishment and the support of a stable EBV infection (Tsang *et al.*, 2010, 2012; Yip *et al.*, 2013), both common events in NPC and GC development (Lee *et al.*, 2008; Lo *et al.*, 2004, 2012; Nagini, 2012; Qu *et al.*, 2013). Thus, cells with enhanced living potential seem to be more susceptible to EBV infection.

EBV itself has the ability to induce telomerase activity in B-cells (Jeon *et al.*, 2009; Kataoka *et al.*, 1997; Sugimoto *et al.*, 2004). Key player for such telomerase activity induction is LMP1, the major EBV-encoded oncogene. Notably, LMP1 induces telomerase activity via NF-κB activation in B-cells and after ectopic expression in epithelial cells (Ding *et al.*, 2005; Mei *et al.*, 2006; Terrin *et al.*, 2008). Additionally, it was shown that hTERT induction contributes to EBV maintenance by induction of latent and down-regulation of lytic EBV gene expression in early-passage infected B lymphocytes (Terrin *et al.*, 2007). Moreover, hTERT inhibition might promote lytic EBV replication in EBV-immortalized and fully transformed B cells (Giunco *et al.*, 2013). Nevertheless, the impact of hTERT expression and telomerase activity on the EBV infection in epithelial cells remains to be elucidated.

Following the current hypothesis that acquired genetic and epigenetic alterations within epithelial stem cells and transiently proliferating cell compartments generates an environment that enables a persistent EBV infection, we aimed to investigate the impact of hTERT expression and telomerase activity on the infection of epithelial cells. We hypothesized that increased telomerase activity in epithelial cells contributes also to EBV maintenance by supporting their infection. To prove our hypothesis, we generated epithelial model cell lines (i) with increased telomerase activity, by ectopic expression of hTERT, and (ii) with lowered telomerase activity, by ectopic expression of a catalytically inactive DNhTERT. Subsequently, we exposed the model cell lines to EBV assessed the frequencies of infection by flow cytometry and EBV gene expression by RT-qPCR.

IX.1.3 Material and Methods

Cells and Viruses

As epithelial model cell lines we used the nasopharyngeal carcinoma (NPC) cell line HONE-1 (Glaser *et al.*, 1989), maintained in RPMI-1640 (Sigma-Aldrich, Buchs, Switzerland), the gastric carcinoma cell line AGS (Barranco *et al.*, 1983), maintained in HAM's F-12 (Sigma-Aldrich) and the human embryonic kidney cell line HEK293 (Graham *et al.*, 1977), maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich). All media were supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Sigma-Aldrich), 1% L-Glutamine and 1% Penicillin/Streptomycin (Gibco, Zug, Switzerland).

The EBV producer cell line HEK293-rB95-8 (Delecluse *et al.*, 1998), kindly provided by Wolfgang Hammerschmidt, was maintained in DMEM (Sigma-Aldrich) supplemented with heat inactivated 10% FBS, 1% L-Glutamine, 1% Penicillin/Streptomycin, 100 µg/ml Hygromycin B (HygroGOLD; InvivoGen). Virus containing supernatants were obtained as described elsewhere (Feederle *et al.*, 2007). Briefly, 80-90% confluent 10 cm dishes of HEK293-rB95-8 cells were transfected with 2 µg each of expression plasmids encoding *BZLF1*, to induce lytic replication, and *BALF4* to optimize gp110 levels on the viral surface (Neuhierl *et al.*, 2002) using Metafectene (Biontex, Martinsried/Planegg, Germany). Four hours after transfection, the transfection mixture was replaced by fresh supplemented DMEM without Hygromycin B. Three to four days after transfection, supernatants were harvested, cleared by centrifugation at 4°C with 1.000 x g for 15 min, filtered through a 0.45 µm filter and stored at -80°C. The number of infectious EBV units was determined as described by Dirmeier *et al.* (2003) and virus titers are given as multiplicity of infection (MOI) and defined as infectious units/cell (Dirmeier *et al.*, 2003; Feederle *et al.*, 2007).

Generation of hTERT- and DNhTERT-overexpressing epithelial cell lines

To generate hTERT-overexpressing we employed the expression vector pWZL-Blast-Flag-HA-hTERT (Maida *et al.*, 2009), kindly provided by William C. Hahn. To generate cells expressing the dominant negative hTERT (DNhTERT) we exchanged the hTERT insert from pWZL-Blast-Flag-HA-hTERT with the DNhTERT mutant from the expression vector pBABE-DNhTERT (Hahn *et al.*, 1999), kindly provided by Bob Weinberg, using the *Eco*RI/*Sal*I restriction sites. Empty control vector was generated by excision of the hTERT insert from pWZL-Blast-Flag-HA-hTERT, using the *Xho*I/*Sal*I restriction sites and

subsequent re-ligation. We then transfected either $1x10^6$ HONE-1, AGS or HEK293 cells with the hTERT-, DN-hTERT or the empty vector (later referred as HONE-1, AGS or HEK293-EV, -hT and -DN), respectively, using Metafectene and 2 days post transfection we selected for resistant cells and maintained the cells with the addition of 10 µg/ml Blasticidin (InvivoGen) to the normal growth medium to establish stable cell lines.

Gene expression analysis by RT-qPCR

Gene expression was determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using specific primers and probes for the housekeeping gene *hydroxymethylbilane synthase* (*HMBS*), the non-coding EBV encoded RNA EBER1, the latency associated EBV genes *EBNA1*, *EBNA2*, for *LMP1* and for the two genes related to the lytic replication cycle of EBV, *BZLF1* and *BXLF2*, as described earlier (Dorner *et al.*, 2008; Ladell *et al.*, 2007). Gene expression of *hTERT* and *DNhTERT* was determined using a pre-validated primer/probe assay (Hs00972656; Applied Biosystems, Zug, Switzerland). Therefore, total RNA was isolated 72 h post infection (p.i.) using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland), followed by DNase treatment (DNA-free Kit; Ambion, Zug, Switzerland) and cDNA synthesis from 0.5 µg RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturers instructions. All reactions were performed in triplicates for each condition and gene on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the TaqMan Gene Expression Master Mix (Applied Biosystems). Cycling conditions were as follows: a 10 min denaturation step at 95°C was followed by 40 cycles of denaturation for 15 s at 95°C and annealing and synthesis for 1 min at 60°C. Results were analyzed with the software SDSv2.3 (Applied Biosystems) and gene expression was calculated relative to the housekeeping gene *HMBS* using the 2^dCt method. Cycle threshold (Ct) values from technical replicates with standard deviations (SD) > 0.5 were excluded from gene expression calculations. Ct values above 36, resulting in relative gene expression levels below 0.001, defined the limit of detection since these values become unreliable above this threshold.

Western Blot analysis

To determine Telomerase protein levels by western blot analysis, whole-cell extracts were prepared from $1x10^6$ cells using RIPA buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate) supplemented with complete mini protease

inhibitor cocktail (Roche, Rotkreuz, Switzerland). After determination of the protein concentration using the Pierce BCA Protein Assay Kit (Thermo Scientific, Wohlen, Switzerland), protein extracts were separated on 4-12% NuPAGE Bis-Tris Precast gels (Invitrogen, Zug, Switzerland) and proteins were semi-dry transferred for 45 min with 25 V on nitrocellulose membranes (Optitran BA-S83; Whatman, Wohlen, Switzerland). hTERT and DNhTERT protein was probed with the primary Telomerase reverse Transcriptase antibody Y182 (1:500; Novus Biologicals, Luzern, Switzerland) and as loading control β-Actin was probed with the primary β-Actin antibody (dilution 1:5000; #4967, Cell Signaling, Allschwil, Switzerland). Primary antibodies were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000; #7074, Cell Signaling). Signals were obtained by incubation with the SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) following manufacturer instructions and visualized on the Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was determined using the TRAPeze Telomerase Detection Kit (S7700; Millipore, Zug, Switzerland) following manufacturers instructions with following modifications. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). The reactions were carried out with 1:50 diluted cell lysates, corresponding to 100 cells. Telomerase extension reaction was performed at 30°C for 30 min followed by 2 min denaturation at 94°C and addition of 2 units *Taq* Polymerase per reaction. Amplification of the telomeric repeats was done in 30 cycles including denaturation at 94°C for 5 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min and a final single-step elongation at 72°C for 5 min. TRAP reactions were separated on 10% TBE gels (Invitrogen) and products were visualized after staining with SYBR Green I nucleic acid gel stain, according to manufacturers instructions (Sigma-Aldrich) using the GeneFlash gel documentation system (Syngene, Châtel-St-Denis, Switzerland).

Direct infection of epithelial cells with cell-free virus by spinoculation

For the direct infection of epithelial cells with cell-free virus we employed an adapted spinoculation protocol to achieve measurable rates of infection (Dorner *et al.*, 2008). Briefly, $1x10^5$ cells were seeded in 12-well plates (TPP, Trasadingen, Switzerland) and incubated over night at 37°C with 5% CO_2. Target cells were then infected, by adding cell-free rB95-8

supernatant with varying MOIs, as indicated, to the target cells in a total volume of 500 µl to ensure equal virus concentrations. Then cells were centrifuged for 1 h at 32°C with 800 x g, supernatant was aspirated, replaced by 1 ml fresh medium and incubated for 72 h at 37°C with 5% CO_2. To determine infection frequencies, cells were detached 72 h p.i., using 0.25% Trypsin-EDTA (Gibco), washed with 1x phosphate buffered saline (PBS; Gibco), stained with the cell viability dye 7-Amino-Actinomycin D (7-AAD; BD Bioscience, Allschwil, Switzerland), to exclude non-viable cells, according to manufacturer's instructions. After one wash with 1x PBS the amount of GFP positive (infected cells) was determined by flow cytometry using the FACS Canto II (BD Bioscience) within the living cell population. Mock infections of each cell line were performed without virus and the amount of GFP positive cells detected as background signals were subtracted (-mock) from corresponding infections.

Statistical analysis

Data sets were tested for statistical significance as indicated using Prism6 (GraphPad, La Jolla, CA, USA) and *P* values <0.05 were regarded as statistically significant.

IX.1.4 Results

Generation of epithelial cell lines with increased telomerase expression or expression of the dominant negative telomerase mutant

To address the question whether hTERT expression level contributes to EBV infection in epithelial cells, we first established an *in vitro* model using three EBV-negative epithelial cell lines: the NPC cell line HONE-1, the gastric carcinoma cell line AGS and the standard epithelial model cell line HEK293. The cells were transfected either with an expression vector encoding for hTERT (hT), the rate limiting component of the human telomerase complex, the telomerase reverse transcriptase, or for its catalytically inactive mutant DNhTERT (DN) (Hahn *et al.*, 1999), respectively. Cells transfected with the empty vector (EV) served as control. After transfection, cells were selected for stable integration.

To confirm overexpression in hTERT and DNhTERT transfected cells, we first investigated gene and protein expression (Fig. 1). Compared to EV control cells, we observed increased *hTERT* and *DNhTERT* gene expression in HONE-1 cells by 92.95 ± 13.07 and 106.03 ± 30.16 fold, respectively; in AGS cells 20.87 ± 4.71 and 25.69 ± 9.14 fold, respectively; and in HEK293 cells 162.72 ± 28.84 and 153.34 ± 31.82, respectively (Fig. 1A).

Western blot quantification of protein expression (Fig. 1B) indicated increased protein expression of hTERT and DNhTERT in HONE-1, AGS, and HEK293 cells in good agreement with the increased gene expression.

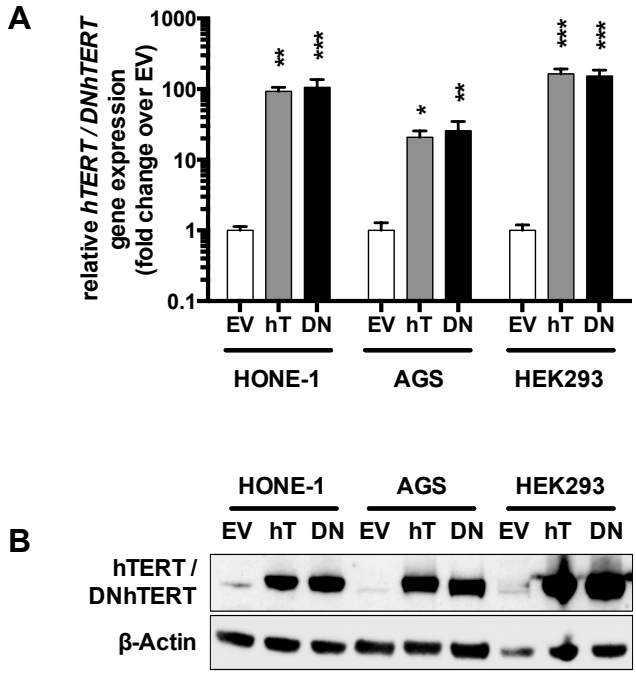

Figure 1: hTERT and DNhTERT expression in epithelial cell lines. A) hTERT and DNhTERT mRNA levels were determined in empty vector control (EV; white), in hTERT (hT; grey) and dominant negative hTERT (DN; black) overexpressing cells by RT-qPCR relative to *HMBS* and shown as fold change over EV. Data is shown as Mean ±SD of 3 independent experiments. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ (ordinary one-way ANOVA; Dunnett's multiple comparison test). B) Protein expression was confirmed by western blot using β-Actin as loading control.

Increased hTERT expression leads to increased telomerase activity, which is suppressed upon expression of the catalytically inactive, dominant negative mutant DNhTERT

After having confirmed overexpression of hTERT and DNhTERT in the engineered cell lines, we asked how hTERT and DN-hTERT expression impacts on telomerase activity. To assess telomerase activity in the epithelial model cell lines, we employed the Telomeric Repeat Amplification Protocol (TRAP assay). Figure 2A shows the results of a representative assay. Endogenous telomerase activity was readily detected in all three EV control cell lines (Fig. 2B). Nevertheless, compared to the robust increase of gene and protein expression, the ectopic expression of hTERT led to a modest increase of telomerase activity in HONE-1-hT (104.8% ± 15.8 of EV control) and HEK293-hT (115.0% ± 0.1 of EV control) cells compared to the EV controls. This might be due to an endogenous already high telomerase activity of wild type cancer cells leading to non-optimal assay conditions as exemplified by the weak standard internal control (S-IC) in HEK293-EV and -hT cells due to the competitive nature of the assay (Fig. 2A). The telomerase activity in AGS-hT cells was increased to 222.5% ± 49.9 of EV-control cells. The expression of DNhTERT led to suppression of telomerase activity below endogenous levels in all three cell lines with the strongest reduction observed in

HONE-1-DN cells (24.1% ± 18.0 of EV control) followed by HEK293-DN cells (52.3% ± 0.02 of EV control) and AGS-DN cells (60.1% ± 12.2 of EV control). Taken together, expression of hTERT or DNhTERT was significantly increased in all stable cell lines. However, telomerase activity was markedly increased only in AGS-hT cells, as measured by the TRAP assay. The expression of the DNhTERT led to consistent decrease of telomerase activity of about 50% in all three stable cell lines.

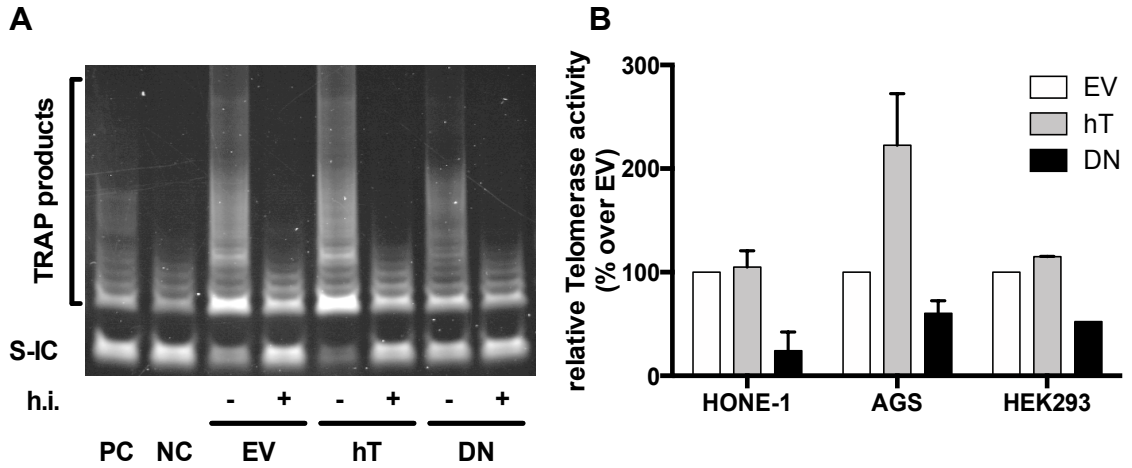

Figure 2: Relative telomerase activity in epithelial cell lines. Telomerase activity in the epithelial cell lines HONE-1, AGS and HEK293 was determined by TRAP assay (A; representative assay) in empty vector (EV) control, in hTERT (hT) and dominant negative hTERT (DN) overexpressing cells and shown relative to EV (B). Data is represented as Mean ± SD from triplicate measurements; S-IC = standard internal control; PC = positive control; NC = negative control; h.i. = heat inactivated.

Increased hTERT expression and activity correlates in part with the infection of epithelial cells by EBV

After establishment of stably hTERT- and DNhTERT-overexpressing cell lines we investigated the impact of hTERT expression and telomerase activity on the infection of these epithelial model cell lines. Therefore, we performed infection studies using a recombinant, wild type EBV strain, rB95-8, that carries a green fluorescent protein (GFP) and allows identification of infected cells by determining GFP expression (Delecluse *et al.*, 1998). We infected the cells using a spinoculation protocol (Dorner *et al.*, 2008) with varying MOIs and determined the amount of infected cells 72h p.i. by flow cytometry as shown in Figure 3.

The EBV-negative cell line HONE-1 originates from an EBV-positive NPC that has lost EBV *in vitro* (Glaser *et al.*, 1989; Yao *et al.*, 1990). Surprisingly, there was no measurable infection (<0.05% GFP positive cells) of the three stable HONE-1 cell lines (Fig.

3A), indicating that the infection of epithelial cells may be dependent on the cellular context and on clonal effects *in vitro* as suggested by Glaser *et al*. (Glaser *et al.*, 1989).

AGS cells showed in general low EBV infection frequencies, depending on MOI, ranging from 0.02 ± 0.03 to 2.51% ± 0.33 (Fig. 3B). Using MOIs of 0.5 and 1, we observed significantly increased infection frequencies of AGS-hT (0.47% ± 0.05 and 0.46% ± 0.03) compared to AGS-EV control cells (0.30% ± 0.008 and 0.20% ± 0.09). Surprisingly, with further increase of EBV dose (MOI 2.5) AGS-hT cells showed a lower infection frequency compared to AGS-EV cells (2.06% ± 0.16 vs. 2.51% ± 0.33) but the difference was not significant. The expression of the dominant negative hTERT mutant and therefore the suppression of telomerase activity in AGS-DN cells inhibited the infection of these cells completely, at least for MOIs of 0.5 (0.02% ± 0.07) or 1 (0.02% ± 0.03). The infection with MOI 2.5 resulted in a further increased infection frequency of AGS-DN cells, but that still was lower compared to that of EV control cells (0.55% ± 0.14 vs. 2.51% ± 0.33; $p<0.0001$). These results indicate that the infection of AGS cells by EBV at low MOIs is dependent on telomerase activity and suggest a contribution of telomerase activity to increased susceptibility to infection by EBV or more likely to support of EBV replication and maintenance in AGS cells.

The infection frequencies in HEK293 cells were in general quite robust and ranged from 1.85% ± 0.2 to 10.84% ± 0.93 as shown in Figure 3C. Upon infection with MOI 0.5 HEK293-EV, -hT and –DN cells showed infection frequencies of 1.85% ± 0.2, 2.05% ± 0.11 and 2.23% ± 0.19, respectively, without significant difference between various hTERT expression and telomerase activity levels. Upon infection with higher MOIs of 1 and 2.5 we did see increased infection frequencies for HEK293-hT (3.96% ± 0.09 and 10.84% ± 0.93) cells compared to the EV control cells (2.32% ± 0.12 and 7.36% ± 0.54). To our surprise, we found as well significantly increased infection rates of HEK293-DN cells (3.89% ± 0.19 and 9.11% ± 0.49) compared to HEK293-EV control cells upon an infection with MOI 1 and 2.5. These results indicate that the infection of HEK293-hT is dependent on telomerase activity. The infections of HEK293-DN cells additionally indicate that activity-independent telomerase functions might potentially be involved in the infection of epithelial cells.

Taken together, we observed, at least partially, augmented EBV infection frequencies of epithelial cells with increased hTERT expression levels and therefore increased telomerase activity and decreased infection frequencies in cells with reduced telomerase activity. These

results suggest that cells with higher telomerase activity are either more susceptible to an infection by EBV or that increased telomerase activity supports EBV maintenance in epithelial cells and that activity-independent telomerase functions might potentially contribute to the infection of epithelial cells by EBV. These effects, however, seemed to be strongly dependent on the amount of EBV and especially on the cellular background, since HONE-1 cells were in general not susceptible to an infection by EBV and the infection frequencies of HEK293 cells were consistently at least 2-fold higher compared to AGS cells at the corresponding MOI.

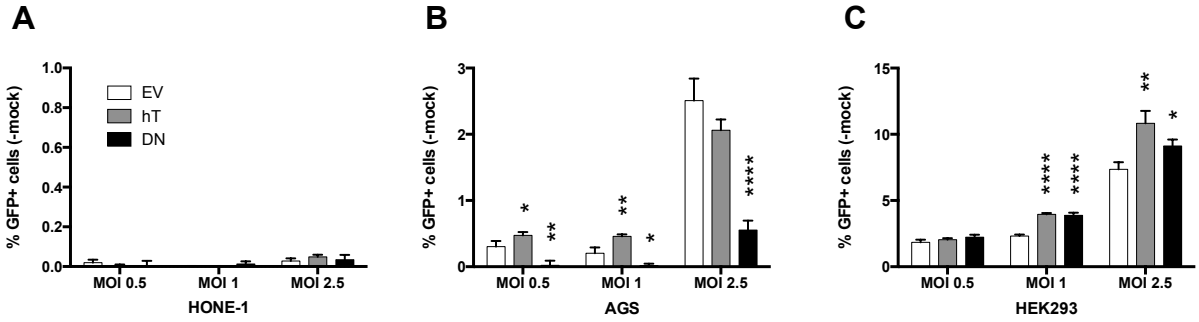

Figure 3: Infection frequencies of epithelial cell lines. The amount of infected (in %GFP positive) HONE-1 (A), AGS (B) and HEK293 (C) cell lines was determined within the living cell (7-AAD negative) population by flow cytometry 72h p.i. after subtraction of the background signal obtained from mock infected cells (-mock). Data is represented as Mean ± SD of 3 independent infections. EV control cells = white; hT = grey; DN = black; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$ (ordinary one-way ANOVA; Dunnett's multiple comparison test).

Telomerase activity supports infection of epithelial cell lines by EBV by enhancing EBV gene expression

Upon infection with rB95-8, the expression of GFP is driven by the constitutive CMV promoter (Delecluse *et al.*, 1998). GFP expression does not necessarily indicate infection of the cell since it might be expressed independently of EBV gene expression or replication. As a second indication for an ongoing infection of these cells and to further investigate the impact of telomerase activity, we determined the EBV gene expression in our epithelial model cell lines. Therefore, we investigated the expression of the non-coding RNA EBER1, of three latency-associated genes *EBNA1*, *EBNA2* and *LMP1* as well as the immediate-early lytic gene *BZLF1* and the late lytic gene *BXLF2*. EBV gene expression was determined in AGS cells and HEK293 cells upon infection at MOI 2.5 as shown in Figure 4A and B, respectively, since we obtained the most robust infections at this MOI and the infection in HONE-1 cells was at the limit of detection or below.

Expression of all EBV genes tested in our panel and of EBER1 was detected in cells from all six cell lines. The most abundant transcripts in the AGS cells were *EBNA1* and in the HEK293 cells EBER1 (relative expression data not shown).

Increased hTERT expression and telomerase activity in AGS-hT cells led to increased transcription of all EBV genes including EBER1 with the exception of *BZLF1* that was reduced to 0.65-fold of EV control cells. *LMP1* showed the strongest increase of expression with a 5.43-fold up-regulation compared to EV control cells. The expression of *EBNA2* (2.5-fold) and *BXLF2* (1.97-fold) was as well significantly up-regulated in comparison to AGS-EV cells. Suppression of telomerase activity in AGS-DN cells resulted in lower expression of all genes except of *LMP1* compared to EV control cells. *BZLF1* expression (0.08-fold) was significantly lower in AGS-DN and *LMP1* (1.02-fold) was transcribed at a similar level in comparison to AGS-EV cells. The expression of hTERT in HEK293-hT cells led as well to the up-regulation of all EBV genes tested and *LMP1* showed the strongest and most significant up-regulation of 2.74-fold over EV control cells. Additionally, expression of *EBNA1* (1.69-fold), *EBNA2* (1.97-fold) and *BXLF2* (1.99-fold) was significantly up-regulated in HEK293-hT cells compared to HEK293-EV cells. EBV gene expression in HEK293-DN cells was comparable to EV control cells, showing as well that increased telomerase activity contributes to EBV gene expression in HEK293 cells.

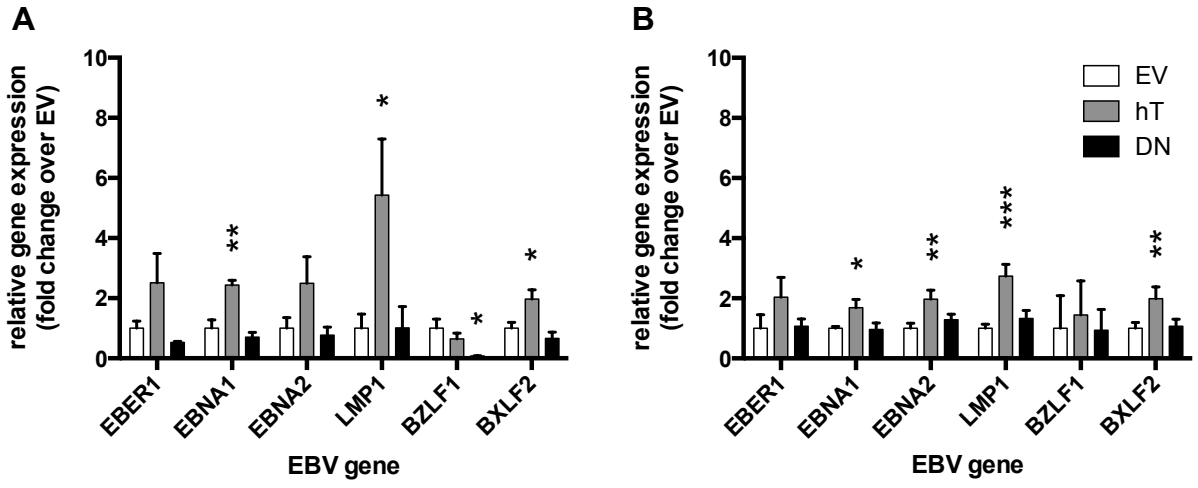

Figure 4: EBV gene expression in epithelial cells upon infection. EBV gene expression was determined in AGS (A) and HEK293 (B) empty vector control (EV; white), in hTERT (hT; grey) and in dominant negative hTERT (DN; black) cells, respectively, 72h p.i. at MOI 2.5 by RT-qPCR relative to *HMBS* and shown as fold change over EV control. Data is represented as Mean ± SD of 3 independent infections; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ (ordinary one-way ANOVA; Dunnett's multiple comparison test).

Taken together, these results indicate that the expression of EBV genes is depending on telomerase activity in our epithelial model cell lines AGS and HEK293. However, only in AGS-DN cells the suppression of telomerase activity did lead to down-regulation of EBV gene expression but not to increased expression of *BZLF1* as shown by others (Giunco *et al.*, 2013; Terrin *et al.*, 2007) in EBV positive Burkitt's lymphoma and lymphoblastoid cell lines. This suggests a contribution of telomerase activity to latent EBV replication and maintenance by enhancing EBV gene expression in epithelial cells.

IX.1.5 Discussion

In this study we generated *in vitro* model systems to study the impact of increased hTERT expression and telomerase activity on the infection of epithelial cells by EBV. We found that increased telomerase activity contributes to the EBV infection of epithelial cells. Our data indicate that i) increased telomerase activity can contribute to the infection of epithelial cells; ii) increased telomerase activity can generate an environment facilitating EBV replication as shown at gene expression level with increased EBV gene expression in hTERT-overexpressing cells, and iii) the infection of epithelial cells is strongly dependent on the cellular context and might be even more dependent on clonal effects *in vitro*. This is the first study showing that EBV gene expression is enhanced by increased telomerase activity, mediated through ectopic expression of hTERT.

We increased hTERT expression and telomerase activity in the epithelial model cell lines by transfection and selection for stable integration. In contrast to the strong increase of hTERT expression at mRNA and protein level in all three hT-cell lines, telomerase activity in HONE-1-hT and HEK293-hT was slightly increased, most likely due to the relatively high endogenous telomerase activity. These results are in line with the findings of Hahn and colleagues, showing slightly increased telomerase activity upon expression of hTERT in telomerase positive cancer cell lines (Hahn *et al.*, 1999)

Although suppression of telomerase activity below endogenous levels was achieved in all DN-cell lines, we could not fully block telomerase activity through the expression of the catalytically inactive DNhTERT mutant and did not observe an inhibiting effect on cell proliferation as in contrast demonstrated by Hahn *et al.* (Hahn *et al.*, 1999). All stable DNhTERT expressing cell lines showed similar growth behavior in culture compared to their corresponding EV control and hTERT cells. However, we did not select for single cell clones

but rather used the whole pool of stably transfected cells for our experiments. This might have resulted in more heterogeneous expression levels within the pool of stably transfected cells compared to clonal populations. Cell clones with sufficient amounts of DNhTERT, to completely block telomerase activity, might indeed stop proliferating and eventually become apoptotic (Hahn *et al.*, 1999). These cells are therefore potentially lost during the selection procedure. As shown by Hahn and colleagues (Hahn *et al.*, 1999), we did as well observe single cells with characteristic large and flattened crisis associated morphology in the stably DNhTERT-transfected cell lines, especially in AGS-DN cells, indicating presumably cells that stopped proliferating. However, the number of cells with this appearance was not significantly different from corresponding EV or hT cultures.

The infection studies of the epithelial model cell lines revealed a quite heterogeneous infection pattern. First, infection of HONE-1 cells was not achieved in our experimental setup, which is not very surprising since it has been shown that the originally EBV-harboring cell line HONE-1 had lost its susceptibility to direct infection *in vitro* (Glaser *et al.*, 1989), indicating that the infection of epithelial cells may depend on the cellular context and on clonal effects. Additionally, Tsang and colleagues (Tsang *et al.*, 2010) showed infection of HONE-1 cells by cell-to-cell contact with EBV positive and lytically induced BL cells, indicating a distinct mode of infection in nasopharyngeal cells. For further studies it will be important to employ a cell-to-cell contact infection protocol to investigate the impact of telomerase activity on the EBV infection of HONE-1 cells.

The infection of AGS and HEK293 cells confirmed the strong dependence on the cellular background since infection frequencies in HEK293 cells were in general at least 2-fold higher then in AGS cells. We observed that in AGS cells hTERT expression and therefore increased telomerase activity can contribute to modulate EBV infection, as shown by increased infection frequencies in AGS-hT cells at low MOIs and inhibited or impeded infections in cells expressing the catalytically inactive DNhTERT mutant. These results suggest a contribution of telomerase activity to either increase susceptibility to infection or to foster the establishment of an environment supporting EBV replication and maintenance. Increased infection frequencies of HEK293-DN cells compared to EV control cells additionally indicate that hTERT activity independent effects might also influence infection rates. For example hTERT was shown to act as transcriptional modulator of the Wnt/β-catenin pathway (Park *et al.*, 2009) that in turn might modulate expression of a putative receptor for EBV binding or entry into epithelial cells. To add another level of complexity to

the whole story, the infection efficiency itself and the effect of hTERT on the infection are dependent on the viral dose. Probably, the maximum level of infection of AGS cells was exceeded with MOI 2.5, as suggested by the decreasing differences observed between AGS-EV and AGS-hT and by the increased infection frequencies of AGS-DN cells. We expected that infection studies with higher MOIs on HEK293 cells would have revealed pronouncedly enhanced infection frequencies due to hTERT expression and telomerase activity in these cells, but this was not the case. We feel that in this context, the gastric carcinoma cell line AGS represents a more physiological model better reflecting the *in vivo* situation than HEK293 cells, given that GC may be associated with EBV.

The investigation of EBV gene expression in AGS and HEK293 cells revealed that the infection is more of latent nature although we detected expression of *BZLF1*, the master regulator of lytic EBV replication induction, and a certain amount of *BXLF2* gene expression, a late lytic EBV gene. We expected lower expression of *BZLF1* in hTERT-overexpressing cells, and this was observed in AGS-hT but not in HEK293-hT cells. Moreover, we expected increased expression in cells with suppressed telomerase activity since it was shown that hTERT expression inhibits lytic EBV replication and that hTERT silencing leads to increased *BZLF1* gene expression in B-cells (Giunco *et al.*, 2013; Terrin *et al.*, 2007). However, we did not observe increased *BZLF1* expression upon suppression of telomerase activity, probably because of the low infection frequencies in AGS-DN cells and the relatively high remaining endogenous telomerase activity in HEK293-DN cells. On the other hand, *BZLF1* expression was reported in hTERT-immortalized primary NPE cells but was rapidly lost early after infection and was very low or undetectable in these stably EBV-infected NPE cell lines (Tsang *et al.*, 2010, 2012; Yip *et al.*, 2013). We would expect this to happen as well with further propagation of our infected cells. Except *BZLF1,* all other EBV genes were up-regulated in both AGS-hT and HEK293-hT cells, while suppression of telomerase activity led to lower expression of EBV genes at least in AGS-DN cells. This confirms that telomerase activity contributes to the establishment and maintenance of the EBV infection in epithelial cells through up-regulation mainly of latency associate EBV genes.

A major limitation of our study is that we used transformed cell lines instead of primary epithelial cells to establish the model system. The vast majority of cancer cell lines as well as primary tumors possess a certain amount of telomerase activity, which is a hallmark of cancer (Hanahan & Weinberg, 2011, 2000). So do our epithelial model cell lines, since hTERT was already endogenously expressed and telomerase activity was detectable in these

model cell lines. However, we are convinced that at least the cell line AGS is suitable to study the impact of telomerase activity on the infection of epithelial cells since they posses a relatively low endogenous telomerase activity and other common features of NPC and GC like cyclin D1 expression and lack of p16 expression (Li *et al.*, 2012; Mattioli *et al.*, 2007). Another limitation of this study might be that we only used one virus strain for the infection of epithelial cells. Very recently, another EBV strain called M81, isolated from a Chinese patient with NPC, was described (Tsai *et al.*, 2013). In contrast to the common laboratory wild type EBV strains B95-8 and Akata, M81 exhibits an enhanced tropism for epithelial cells and a preference for lytic replication but is still able to transform B-cells efficiently. With regard to NPC, using this strain for the infection experiments would be more physiological to study the infection of epithelial cells with enhanced telomerase expression and activity. Additionally, we would expect more robust infection frequencies and EBV gene expression upon infection with M81. This would potentially reveal more pronounced effects of hTERT expression and telomerase activity on the infection of epithelial cells by EBV and its contribution to EBV maintenance.

Our findings contribute to the current hypothesis that EBV may be able to infect epithelial cells but that there are additional factors needed, e.g. chromosomal aberration, genetic and epigenetic alteration or dysregulated cell signaling pathways, to render them permissive for a latent EBV infection and that the infection by EBV is a late event in the development of EBV associated epithelial cell carcinomas (Fukayama & Ushiku, 2011; Zur Hausen *et al.*, 2004; Iizasa *et al.*, 2012; Lo *et al.*, 2012; Tsang *et al.*, 2012; Tsao *et al.*, 2012; Yoshizaki *et al.*, 2013; Young & Rickinson, 2004). Based on this hypothesis and our results we speculate that enhanced EBV gene expression supported by increased telomerase activity drives malignant transformation and tumor progression in epithelial cells. We further hypothesize that hTERT and EBV cooperate in a positive feedback loop since EBV itself can induce telomerase activity. That would lead in turn to promotion of latent EBV infection and the expression of latent gene products, such as LMP1 and LMP2A. The expression of latent proteins will then facilitate rapid clonal expansion and transformation thus favoring further accumulation of genetic alteration and epigenetic changes and ultimately contribute to malignant transformation and tumor progression.

IX.1.6 Acknowledgment

This work was supported by the Swiss National Science Foundation (grant 310030_135028 to D. N.) and the Cancer League of the Canton Zurich (to D. N.).

IX.1.7 References

Barranco, S. C., Townsend, C. M., Casartelli, C., Macik, B. G., Burger, N. L., Boerwinkle, W. R. & Gourley, W. K. (1983). Establishment and characterization of an in vitro model system for human adenocarcinoma of the stomach. *Cancer Res* **43**, 1703–9.

Chan, A. S., To, K. F., Lo, K. W., Mak, K. F., Pak, W., Chiu, B., Tse, G. M., Ding, M., Li, X. & other authors. (2000). High frequency of chromosome 3p deletion in histologically normal nasopharyngeal epithelia from southern Chinese. *Cancer Res* **60**, 5365–70.

Chan, A. S. C., To, K. F., Lo, K. W., Ding, M., Li, X., Johnson, P. & Huang, D. P. (2002). Frequent chromosome 9p losses in histologically normal nasopharyngeal epithelia from southern Chinese. *Int J Cancer* **102**, 300–3.

Crowe, D. L., Nguyen, D. C. & Ohannessian, A. (2005). Mechanism of telomerase repression during terminal differentiation of normal epithelial cells and squamous carcinoma lines. *Int J Oncol* **27**, 847–54.

Delecluse, H. J., Hilsendegen, T., Pich, D., Zeidler, R. & Hammerschmidt, W. (1998). Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc Natl Acad Sci U S A* **95**, 8245–50.

Ding, L., Li, L. L., Yang, J., Tao, Y. G., Ye, M., Shi, Y., Tang, M., Yi, W., Li, X. L. & other authors. (2005). Epstein-Barr virus encoded latent membrane protein 1 modulates nuclear translocation of telomerase reverse transcriptase protein by activating nuclear factor-kappaB p65 in human nasopharyngeal carcinoma cells. *Int J Biochem Cell Biol* **37**, 1881–9.

Dirmeier, U., Neuhierl, B., Kilger, E., Reisbach, G., Sandberg, M. L. & Hammerschmidt, W. (2003). Latent membrane protein 1 is critical for efficient growth transformation of human B cells by epstein-barr virus. *Cancer Res* **63**, 2982–9.

Dorner, M., Zucol, F., Berger, C., Byland, R., Melroe, G. T., Bernasconi, M., Speck, R. F. & Nadal, D. (2008). Distinct ex vivo susceptibility of B-cell subsets to epstein-barr virus infection according to differentiation status and tissue origin. *J Virol* **82**, 4400–12.

Epstein, M. A., Achong, B. G. & Barr, Y. M. (1964). Virus particles in cultured lymphoblasts from Burkitt's Lymphoma. *Lancet* **1**, 702–3.

Feederle, R., Neuhierl, B., Bannert, H., Geletneky, K., Shannon-Lowe, C. & Delecluse, H.-J. (2007). Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection. *Int J Cancer* **121**, 588–94.

Feller, L. L., Khammissa, R. R., Kramer, B. B. & Lemmer, J. J. (2013). Oral squamous cell carcinoma in relation to field precancerisation: pathobiology. *Cancer Cell Int* **13**, 31. Cancer Cell International.

Fukayama, M. & Ushiku, T. (2011). Epstein-Barr virus-associated gastric carcinoma. *Pathol Res Pract* **207**, 529–37. Elsevier GmbH.

Giunco, S., Dolcetti, R., Keppel, S., Celeghin, A., Indraccolo, S., Dal Col, J., Mastorci, K. & De Rossi, A. (2013). hTERT inhibition triggers Epstein-Barr virus lytic cycle and apoptosis in immortalized and transformed B cells: a basis for new therapies. *Clin Cancer Res* **19**, 2036–47.

Glaser, R., Zhang, H. Y., Yao, K. T., Zhu, H. C., Wang, F. X., Li, G. Y., Wen, D. S. & Li, Y. P. (1989). Two epithelial tumor cell lines (HNE-1 and HONE-1) latently infected with Epstein-Barr virus that were derived from nasopharyngeal carcinomas. *Proc Natl Acad Sci U S A* **86**, 9524–8.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59–74.

Griffin, B. D., Verweij, M. C. & Wiertz, E. J. H. J. (2010). Herpesviruses and immunity: the art of evasion. *Vet Microbiol* **143**, 89–100. Elsevier B.V.

Gu, A.-D., Zeng, M.-S. & Qian, C.-N. (2012). The criteria to confirm the role of epstein-barr virus in nasopharyngeal carcinoma initiation. *Int J Mol Sci* **13**, 13737–47.

Hahn, W. C., Stewart, S. A., Brooks, M. W., York, S. G., Eaton, E., Kurachi, A., Beijersbergen, R. L., Knoll, J. H., Meyerson, M. & Weinberg, R. A. (1999). Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* **5**, 1164–70.

Hanahan, D. & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57–70.

Hanahan, D. & Weinberg, R. a. **(2011).** Hallmarks of cancer: the next generation. *Cell* **144**, 646–74. Elsevier Inc.

Zur Hausen, A., van Rees, B. P., van Beek, J., Craanen, M. E., Bloemena, E., Offerhaus, G. J. A., Meijer, C. J. L. M. & van den Brule, A. J. C. (2004). Epstein-Barr virus in gastric carcinomas and gastric stump carcinomas: a late event in gastric carcinogenesis. *J Clin Pathol* **57**, 487–91.

Hislop, A. D., Taylor, G. S., Sauce, D. & Rickinson, A. B. (2007). Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* **25**, 587–617.

Iizasa, H., Nanbo, A., Nishikawa, J., Jinushi, M. & Yoshiyama, H. (2012). Epstein-Barr Virus (EBV)-associated gastric carcinoma. *Viruses* **4**, 3420–39.

Jeon, J.-P., Nam, H.-Y., Shim, S.-M. & Han, B.-G. (2009). Sustained viral activity of epstein-Barr virus contributes to cellular immortalization of lymphoblastoid cell lines. *Mol Cells* **27**, 143–8.

Kataoka, H., Tahara, H., Watanabe, T., Sugawara, M., Ide, T., Goto, M., Furuichi, Y. & Sugimoto, M. (1997). Immortalization of immunologically committed Epstein-Barr virus-transformed human B-lymphoblastoid cell lines accompanied by a strong telomerase activity. *Differentiation* **62**, 203–11.

Kieff, E. D. & Rickinson, A. B. (2007). Epstein-Barr Virus and Its Replication. In *Fields Virol*, 5th edn., pp. 2603–2654. Edited by D. M. Knipe & P. M. Howley. Lippincott Williams & Wilkins.

Kumar, S. K. S., Zain, R. B., Ismail, S. M. & Cheong, S. C. (2005). Human telomerase reverse transcriptase expression in oral carcinogenesis--a preliminary report. *J Exp Clin Cancer Res* **24**, 639–46.

Ladell, K., Dorner, M., Zauner, L., Berger, C., Zucol, F., Bernasconi, M., Niggli, F. K., Speck, R. F. & Nadal, D. (2007). Immune activation suppresses initiation of lytic Epstein-Barr virus infection. *Cell Microbiol* **9**, 2055–69.

Lee, K.-H., Lee, H. E., Cho, S. J., Cho, Y. J., Lee, H. S., Kim, J. H., Nam, S. Y., Chang, M. S., Kim, W. H. & Lee, B. L. (2008). Immunohistochemical analysis of cell cycle-related molecules in gastric carcinoma: prognostic significance, correlation with clinicopathological parameters, proliferation and apoptosis. *Pathobiology* **75**, 364–72.

Li, H. M., Man, C., Jin, Y., Deng, W., Yip, Y. L., Feng, H. C., Cheung, Y. C., Lo, K. W., Meltzer, P. S. & other authors. **(2006).** Molecular and cytogenetic changes involved in the immortalization of nasopharyngeal epithelial cells by telomerase. *Int J Cancer* **119**, 1567–76.

Li, R., Pang, X.-Q., Chen, W.-C., Li, L., Tian, W.-Y. & Zhang, X.-G. (2012). Gastric cancer cell lines AGS before and after CD40 signal activating. *Mol Biol Rep* **39**, 6615–23.

Lo, K. W., To, K. F. & Huang, D. P. (2004). Focus on nasopharyngeal carcinoma. *Cancer Cell* **5**, 423–8.

Lo, K.-W., Chung, G. T.-Y. & To, K.-F. (2012). Deciphering the molecular genetic basis of NPC through molecular, cytogenetic, and epigenetic approaches. *Semin Cancer Biol* **22**, 79–86. Elsevier Ltd.

Maida, Y., Yasukawa, M., Furuuchi, M., Lassmann, T., Possemato, R., Okamoto, N., Kasim, V., Hayashizaki, Y., Hahn, W. C. & Masutomi, K. (2009). An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature* **461**, 230–5.

Mattioli, E., Vogiatzi, P., Sun, A., Abbadessa, G., Angeloni, G., D'Ugo, D., Trani, D., Gaughan, J. P., Vecchio, F. M. & other authors. **(2007).** Immunohistochemical analysis of pRb2/p130, VEGF, EZH2, p53, p16(INK4A), p27(KIP1), p21(WAF1), Ki-67 expression patterns in gastric cancer. *J Cell Physiol* **210**, 183–91.

Mei, Y.-P., Zhu, X.-F., Zhou, J.-M., Huang, H., Deng, R. & Zeng, Y.-X. (2006). siRNA targeting LMP1-induced apoptosis in EBV-positive lymphoma cells is associated with inhibition of telomerase activity and expression. *Cancer Lett* **232**, 189–98.

Nagini, S. (2012). Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention. *World J Gastrointest Oncol* **4**, 156–69.

Neuhierl, B., Feederle, R., Hammerschmidt, W. & Delecluse, H. J. (2002). Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc Natl Acad Sci U S A* **99**, 15036–41.

O'Flatharta, C., Leader, M., Kay, E., Flint, S. R., Toner, M., Robertson, W. & Mabruk, M. J. E. M. F. (2002). Telomerase activity detected in oral lichen planus by RNA in situ hybridisation: not a marker for malignant transformation. *J Clin Pathol* **55**, 602–7.

Park, J.-I., Venteicher, A. S., Hong, J. Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P. & other authors. **(2009).** Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature* **460**, 66–72. Nature Publishing Group.

Patel, V., Iglesias-Bartolome, R., Siegele, B., Marsh, C. A., Leelahavanichkul, K., Molinolo, A. A. & Gutkind, J. S. (2011). Cellular systems for studying human oral squamous cell carcinomas. *Adv Exp Med Biol* **720**, 27–38.

Qu, Y., Dang, S. & Hou, P. (2013). Gene methylation in gastric cancer. *Clin Chim Acta* **424**, 53–65. The Authors.

Rickinson, A. B. & Kieff, E. D. (2007). Epstein-Barr Virus. In *Fields Virol*, 5th edn., pp. 2656–2700. Edited by D. M. Knipe & P. M. Howley. Lippincott Williams & Wilkins.

Shannon-Lowe, C., Adland, E., Bell, A. I., Delecluse, H.-J., Rickinson, A. B. & Rowe, M. (2009). Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification. *J Virol* **83**, 7749–60.

Speck, S. H. & Ganem, D. (2010). Viral latency and its regulation: lessons from the gamma-herpesviruses. *Cell Host Microbe* **8**, 100–15. Elsevier Inc.

Sugimoto, M., Tahara, H., Ide, T. & Furuichi, Y. (2004). Steps involved in immortalization and tumorigenesis in human B-lymphoblastoid cell lines transformed by Epstein-Barr virus. *Cancer Res* **64**, 3361–4.

Takada, K. (2012). Role of EBER and BARF1 in nasopharyngeal carcinoma (NPC) tumorigenesis. *Semin Cancer Biol* **22**, 162–5. Elsevier Ltd.

Taylor, G. S. & Blackbourn, D. J. (2011). Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV. *Cancer Lett* **305**, 263–78. Elsevier Ireland Ltd.

Terrin, L., Dolcetti, R., Corradini, I., Indraccolo, S., Dal Col, J., Bertorelle, R., Bonaldi, L., Esposito, G. & De Rossi, A. (2007). hTERT inhibits the Epstein-Barr virus lytic cycle and promotes the proliferation of primary B lymphocytes: implications for EBV-driven lymphomagenesis. *Int J Cancer* **121**, 576–87.

Terrin, L., Dal Col, J., Rampazzo, E., Zancai, P., Pedrotti, M., Ammirabile, G., Bergamin, S., Rizzo, S., Dolcetti, R. & De Rossi, A. (2008). Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. *J Virol* **82**, 10175–87.

Tsai, M.-H., Raykova, A., Klinke, O., Bernhardt, K., Gärtner, K., Leung, C. S., Geletneky, K., Sertel, S., Münz, C. & other authors. **(2013).** Spontaneous Lytic Replication and Epitheliotropism Define an Epstein-Barr Virus Strain Found in Carcinomas. *Cell Rep* 1–13.

Tsang, C. M., Zhang, G., Seto, E., Takada, K., Deng, W., Yip, Y. L., Man, C., Hau, P. M., Chen, H. & other authors. **(2010).** Epstein-Barr virus infection in immortalized nasopharyngeal epithelial cells: regulation of infection and phenotypic characterization. *Int J Cancer* **127**, 1570–83.

Tsang, C. M., Yip, Y. L., Lo, K. W., Deng, W., To, K. F., Hau, P. M., Lau, V. M. Y., Takada, K., Lui, V. W. Y. & other authors. **(2012).** Cyclin D1 overexpression supports stable EBV infection in nasopharyngeal epithelial cells. *Proc Natl Acad Sci U S A* **109**, E3473–82.

Tsao, S. W., Tsang, C. M., Pang, P. S., Zhang, G., Chen, H. & Lo, K. W. (2012). The biology of EBV infection in human epithelial cells. *Semin Cancer Biol* **22**, 137–43. Elsevier Ltd.

Wu, H.-C., Lin, Y.-J., Lee, J.-J., Liu, Y.-J., Liang, S.-T., Peng, Y., Chiu, Y.-W., Wu, C.-W. & Lin, C.-T. (2003). Functional analysis of EBV in nasopharyngeal carcinoma cells. *Lab Invest* **83**, 797–812.

Yao, K. T., Zhang, H. Y., Zhu, H. C., Wang, F. X., Li, G. Y., Wen, D. S., Li, Y. P., Tsai, C. H. & Glaser, R. (1990). Establishment and characterization of two epithelial tumor cell lines (HNE-1 and HONE-1)

latently infected with Epstein-Barr virus and derived from nasopharyngeal carcinomas. *Int J Cancer* **45**, 83–9.

Yasumoto, S., Kunimura, C., Kikuchi, K., Tahara, H., Ohji, H., Yamamoto, H., Ide, T. & Utakoji, T. (1996). Telomerase activity in normal human epithelial cells. *Oncogene* **13**, 433–9.

Yip, Y. L., Pang, P. S., Deng, W., Tsang, C. M., Zeng, M., Hau, P. M., Man, C., Jin, Y., Yuen, A. P. W. & Tsao, S. W. (2013). Efficient immortalization of primary nasopharyngeal epithelial cells for EBV infection study. *PLoS One* **8**, e78395.

Yoshizaki, T., Kondo, S., Wakisaka, N., Murono, S., Endo, K., Sugimoto, H., Nakanishi, S., Tsuji, A. & Ito, M. (2013). Pathogenic role of Epstein-Barr virus latent membrane protein-1 in the development of nasopharyngeal carcinoma. *Cancer Lett* **337**, 1–7. Elsevier Ireland Ltd.

Young, L. S. & Rickinson, A. B. (2004). Epstein-Barr virus: 40 years on. *Nat Rev Cancer* **4**, 757–68.

IX.2 Manuscript II: TLR9 stimulation in epithelial cells does not drive Epstein-Barr virus into latency

Jürgen Rac[1,2], Florian Haas[1,2], Michele Bernasconi[1,2], Roberto F. Speck[3], David Nadal[1,2,*]

[1]Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital of Zurich, University of Zurich, Zurich, Switzerland

[2]Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland

[3]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, Zurich, Switzerland

*Corresponding author:
David Nadal, M.D.
University Children's Hospital of Zurich
Division of Infectious Diseases and Hospital Epidemiology
Steinwiesstrasse 75
CH-8032 Zurich
Switzerland
E-Mail: david.nadal@kispi.uzh.ch
Phone +41 44 266 7562;
FAX +41 44 266 8072

Manuscript in preparation:

Authors' contributions: J.R. planned, performed and analyzed experiments shown in Figure 1-5 and wrote the manuscript. Remaining authors supervised and supported the study design and revised the Manuscript.

IX.2.1 Abstract

The ubiquitous γ-herpesvirus EBV is very successful in infecting >90% of the human population. EBV is transmitted via saliva to the next susceptible host and thus the pharyngeal cavities are portal of entry and exit for Epstein-Barr virus (EBV). Various microbes with specific pathogen associated molecular patterns (PAMPs) constantly challenge the oral mucosal epithelial tissues lining the pharyngeal cavities. As a first line of defense, the innate immune system recognizes these microorganisms via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Signaling triggered by TLR stimulation results in expression of pro-inflammatory cytokines, chemokines and anti-microbial effector molecules and is therefore contributing to shape the adaptive immunity as a second response to invading pathogens. Apart from the mechanical and physical barrier function of pharyngeal epithelial cells, they express TLRs and are capable of mounting an innate immune response. Thus epithelial cells of the pharyngeal cavity play a pivotal role not only within the life cycle of EBV. Recently we have demonstrated that TLR9 activation can suppress the reactivation from latent to lytic EBV infection in EBV positive Burkitt's lymphoma cells whereas triggering of TLR2 in latently infected lymphoblastoid cell lines elicits lytic EBV replication. However, the impact of activated TLR signaling on the infection of epithelial cells by EBV remains elusive. Therefore, we investigated the effect of TLR9 stimulation on EBV infection of epithelial cells. Here, we show that TLR9 triggering with synthetic ligands has no impact on EBV infection of the stably TLR9-expressing epithelial model cell line HEK293 and its wild type counterpart. Additionally, our results indicate that both cell lines might actually not be suitable as epithelial cell models to study the effect of TLR signaling on the infection of epithelial cells by EBV, but they suggest a contributing role of TLR activation in epithelial cells to EBV reactivation from latency.

IX.2.2 Introduction

Epstein-Barr virus (EBV) is a member of the γ-herpesvirus family that is transmitted via saliva between human hosts (Rickinson & Kieff, 2007). The infection by EBV is usually asymptomatic if acquired during early childhood and may cause infectious mononucleosis (IM) if acquired later in life (Rickinson & Kieff, 2007). Apart from IM, EBV is irrefutably linked to various lymphoid and epithelial cell malignancies, e.g. Burkitt's lymphoma (BL), gastric carcinoma (GC) and nasopharyngeal carcinoma (NPC) (Rickinson & Kieff, 2007). Following primary infection, EBV persists life-long within the host B cell pool. Once the B

cells are infected, EBV establishes a latent infection with limited gene expression and therefore a maximum level of immune evasion (Griffin *et al.*, 2010; Hislop *et al.*, 2007; Kieff & Rickinson, 2007; Speck & Ganem, 2010; Taylor & Blackbourn, 2011). Nevertheless, EBV has to undergo lytic replication to produce infectious progeny virus that can be shed into saliva. Lytic EBV replication potentially takes place within permissive epithelial cells of the oropharynx (Rickinson & Kieff, 2007).

As EBV transmission occurs via saliva, the pharyngeal cavities are the portal of entry and exit for EBV (Rickinson & Kieff, 2007). Thus, the pharyngeal epithelium is the first barrier that EBV has to overcome to get access to the B lymphocytes, its target cells for life-long persistence. Various microorganisms constantly challenge the pharyngeal epithelium (Diamond *et al.*, 2008; Feller *et al.*, 2013). Thus, innate immune mechanisms play an important role in the local defense in addition to the mechanical and physical barrier function of epithelial cells. Microbial invaders own specific pathogen associated molecular patterns (PAMPs) that are sensed by so called pattern recognition receptors (PRRs). Amongst these, Toll-like receptors (TLRs) were first PRRs to be identified and are fundamental sensors recognizing a wide range of PAMPs (Kawai & Akira, 2011).

Several studies have shown that epithelial cells, including those of tonsils and upper airways, express TLRs and are capable of mounting innate immune responses (Claeys *et al.*, 2003; Hornef & Bogdan, 2005; Lange *et al.*, 2009a; Sha *et al.*, 2004; Swaminathan *et al.*, 2013; Vandermeer *et al.*, 2004), which influence and prime the subsequent adaptive immunity. EBV itself was shown to activate and modulate TLR2, 3, 7, and 9 signaling in various cell types (Ariza *et al.*, 2009; Fiola *et al.*, 2010; Gaudreault *et al.*, 2007; Iwakiri *et al.*, 2009; Martin *et al.*, 2007; Vérillaud *et al.*, 2012; West *et al.*, 2012). Additionally, we recently demonstrated that activation of TLR9 signaling drives EBV into latency upon primary infection in B cells and inhibits the switch to lytic replication in EBV positive BL cells (Ladell *et al.*, 2007; Zauner *et al.*, 2010). More recently, our group demonstrated that in contrast to TLR9 activation, ligation of TLR2 with heat-killed group A streptococci (GAS) can induce lytic EBV reactivation in latently infected lymphoblastoid cell lines (LCLs), established from tonsillar mononuclear cells (TMCs) (Ueda *et al.*, 2014). However, the impact of TLR signaling on EBV infection in epithelial cells remains elusive. Therefore, we employed two epithelial model cell lines to investigate the influence of TLR9 signaling on the infection of epithelial cells by EBV.

IX.2.3 Material and Methods

Cells and Viruses

As epithelial model cell lines we employed the human embryonic kidney cell line HEK293 (Graham *et al.*, 1977) and HEK293XL-hTLR9-HA cells from InvivoGen (Toulouse, France). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Buchs Switzerland), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Sigma-Aldrich), 1% L-Glutamine and 1% Penicillin/Streptomycin (Gibco, Zug, Switzerland). HEK293XL-hTLR9-HA cells are engineered to stably express human TLR9 gene with an influence hemaglutinine (HA) tag and are therefore maintained with the addition of 10 µg/ml Blasticidin (InvivoGen) in the growth medium. HEK293 and HEK293XL-hTLR9-HA cells are later referred as 293-WT and 293-TLR9, respectively.

The EBV producer cell line HEK293-rB95-8 (Delecluse *et al.*, 1998), kindly provided by Wolfgang Hammerschmidt, was maintained in DMEM (Sigma-Aldrich) supplemented with heat inactivated 10% FBS, 1% L-Glutamine, 1% Penicillin/Streptomycin, 100 µg/ml Hygromycin B (HygroGOLD; InvivoGen). Virus containing supernatants were obtained as described elsewhere (Feederle *et al.*, 2007). Briefly, 80-90% confluent 10 cm dishes of HEK293-rB95-8 cells were transfected with 2 µg each of expression plasmids encoding *BZLF1*, to induce lytic replication, and *BALF4* to optimize gp110 levels on the viral surface (Neuhierl *et al.*, 2002) using Metafectene (Biontex, Martinsried/Planegg, Germany). Four hours after transfection, the transfection mixture was replaced by fresh supplemented DMEM without Hygromycin B. Three to four days after transfection, supernatants were harvested, cleared by centrifugation at 4°C with 1.000 x g for 15 min, filtered through a 0.45 µm filter and stored at -80°C. The number of infectious EBV units was determined as described by Dirmeier *et al.* (2003) and virus titers are given as multiplicity of infection (MOI) and defined as infectious units/cell (Dirmeier *et al.*, 2003; Feederle *et al.*, 2007).

Gene expression analysis by RT-qPCR

Gene expression was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using specific primers and probes for the housekeeping gene *hydroxymethylbilane synthase* (*HMBS*), for *IL-6* and *TNF-α*, the non-coding EBV encoded RNA EBER1 and the viral genes *EBNA1*, *EBNA2*, *LMP1*, *BZLF1* and *BXLF2* as described earlier (Bonanomi *et al.*, 2003; Dorner *et al.*, 2008; Ladell *et al.*, 2007). *TLR1-9* gene

expression was determined using pre-validated primer/probe sets (Hs00413978; Hs00610101; Hs01551078; Hs00152939; Hs00152825; Hs01039989; Hs01933259; Hs00152972; Hs00152973; Assay-on-demand; Applied Biosystems, Zug, Switzerland). Briefly, total RNA was isolated 24 h, 48 h and 72 h post infection (p.i.) using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland), followed by DNase treatment (DNA-free Kit; Ambion, Zug, Switzerland) and cDNA synthesis from 0.5 µg RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturers instructions. All reactions were performed in triplicates for each condition and gene on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the TaqMan Gene Expression Master Mix (Applied Biosystems). Cycling conditions were as follows: a 10 min denaturation step at 95°C was followed by 40 cycles of denaturation for 15 s at 95°C and annealing and synthesis for 1 min at 60°C. Results were analyzed with the software SDSv2.3 (Applied Biosystems) and gene expression was calculated relative to the housekeeping gene *HMBS* using the 2^dCt method. Cycle threshold (Ct) values from technical replicates with standard deviations (SD) > 0.5 were excluded from gene expression calculations. Ct values above 36, resulting in relative gene expression levels below 0.001, defined the limit of detection since these values become unreliable above this threshold.

Western Blot analysis

hTLR9-HA protein expression was verified by western blot analysis. Therefore, whole-cell extracts were prepared from $1x10^6$ cells using Radio-immunoprecipitation assay buffer (RIPA; 50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate) supplemented with complete mini protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). After determination of the protein concentration with the Pierce BCA Protein Assay Kit (Thermo Scientific, Wohlen, Switzerland), protein extracts were separated on 4-12% NuPAGE Bis-Tris Precast gels (Invitrogen, Zug, Switzerland) and proteins were semi-dry transferred for 45 min with 25 V on nitrocellulose membranes (Optitran BA-S83; Whatman, Wohlen, Switzerland). HA-tagged hTLR9 protein was probed with the primary Anti-HA Tag antibody (05-904; 1:1000; Millipore, Zug, Switzerland) and as loading control β-Actin was probed with the primary β-Actin antibody (dilution 1:5000; #4967, Cell Signaling, Allschwil, Switzerland). Primary Anti-HA antibodies were detected using a horseradish peroxidase-conjugated horse anti-mouse IgG (dilution 1:5000; #7076, Cell Signaling) and primary β-Actin antibodies were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000; #7074, Cell Signaling). Signals were

obtained by incubation with the SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) following manufacturer instructions and visualized on the Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Direct infection of epithelial cells with cell-free virus by spinoculation

For the direct infection (DI) of epithelial cells with cell-free virus we employed an adapted spinoculation protocol to achieve measurable rates of infection (Dorner *et al.*, 2008). Briefly, $1x10^5$ cells were seeded in 12-well plates (TPP, Trasadingen, Switzerland) and incubated over night at 37°C with 5% CO_2. Target cells were then infected, by adding cell-free rB95-8 supernatant (MOI 0.5) to the target cells in a total volume of 500 µl to ensure equal virus concentrations. Then cells were centrifuged for 1 h at 32°C with 800 x g, supernatant was aspirated, replaced by 1 ml fresh medium and incubated for 72 h at 37°C with 5% CO_2. To determine infection frequencies, cells were detached 72 h p.i., using 0.25% Trypsin-EDTA (Gibco), washed with 1x phosphate-buffered saline (PBS; Gibco), stained with the cell viability dye 7-Amino-Actinomycin D (7-AAD; BD Bioscience, Allschwil, Switzerland), to exclude non-viable cells, according to manufacturer's instructions. After one wash with 1x PBS the amount of GFP positive (infected cells) was determined by flow cytometry using the FACS Canto II (BD Bioscience) within the living cell population. Mock infections of each cell line were performed without virus and the amount of GFP positive cells detected as background signals were subtracted from corresponding infections.

TLR9 stimulation with synthetic agonists

To trigger TLR9 synthetic human TLR9 agonists containing CpG motifs were employed. Cells were stimulated with 1 µM of either ODN2006 (Class B; CpG-B) or ODN2216 (Class A; CpG-A) both from InvivoGen and the response to TLR9 stimulation was determined after 1 h, 2 h, 4 h and 6 h of stimulation by means of *IL-6* and *TNF-α* gene expression using RT-qPCR. When cells were subjected to DI, cells were stimulated for 2h prior to spinoculation and ODNs were repeatedly added 24h and 48h p.i.. In total 72h p.i., the amount of GFP positive cells was determined by flow cytometry within the living cell population as mentioned above. Cells treated with endotoxin-free water, the solvent for CpG-A and -B, served as mock control for the stimulation.

Statistical analysis

Data is reported as mean ± SD from at least three independent replicates and statistical tests, as indicated, were computed using Prism6 (GraphPad, La Jolla, CA, USA) from at least 3 independent experiments. *P* values <0.05 were regarded as statistically significant.

IX.2.4 Results

TLR expression in wild type and TLR9-engineered HEK293 cells

Wild type HEK293 cells express TLR3 and 5 (Huang *et al.*, 2009; Simon & Samuel, 2007; Smith *et al.*, 2003) and are devoid of TLR2 and 4 (Erridge *et al.*, 2007; Latz *et al.*, 2002). It has been reported as well that TLR7, 8, 9 are not expressed in HEK293 wild type cells (Wang *et al.*, 2006) while others detected TLR9 mRNA in these cells (Assaf *et al.*, 2009).

Since both HEK293 cell lines, 293-WT and 293-TLR9, were obtained from two different sources, we first characterized their endogenous *TLR* gene expression patterns (Fig. 1). We determined *TLR1-9* expression by RT-qPCR relative to the housekeeping gene *HMBS*. 293-WT cells expressed low levels of *TLR1*, *3*, *5*, *6*, *7* and *9* while *TLR2*, *4* and *8* were not detected at all at the RNA level (Fig. 1A). The expression pattern in 293-TLR9 cells was similar but they additionally showed strong expression of *TLR4* (Fig. 1B), which is usually absent in wild type HEK293 cells (Smith *et al.*, 2003). With exception of *TLR9*, which showed a lower expression in 293-TLR9 cells, relative gene expression in 293-TLR9 cells was in general higher compared to 293-WT cells. Endogenous *TLR9* expression was low in both cell lines. These results can be explained by the different origin of the cells or by a clonal selection during the establishment of 293-TLR9 cells.

To confirm ectopical expression of the HA-tagged TLR9 in 293-TLR9 cells we performed western blot analysis (Fig. 1C). As expected, we detected strong expression of TLR9-HA in 293-TLR9 cells and not in 293-WT.

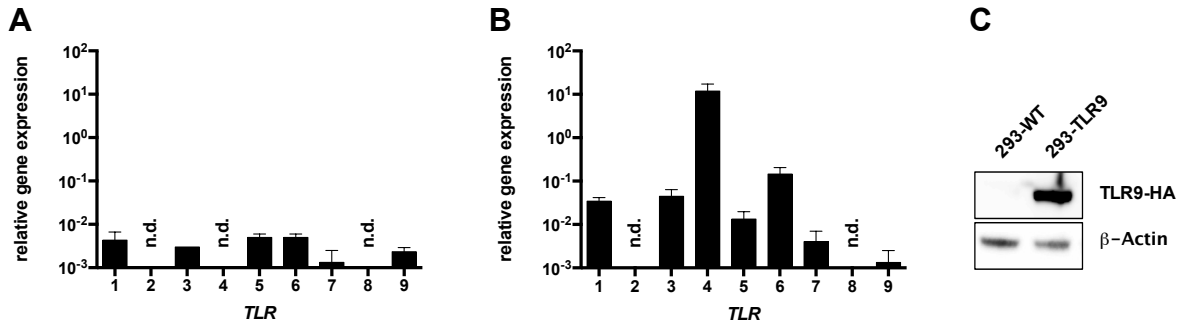

Figure 1: TLR expression in wild type HEK293 and TLR9-engineered HEK293 cells. *TLR1-9* gene expression levels were determined in 293-WT (A) and 293-TLR9 (B) cells by RT-qPCR relative to *HMBS*. Data is represented as Mean ±SD of at least 3 independent replicates; n.d. = not detected. C) Ectopical HA-tagged TLR9 protein expression was confirmed by western blot. β-Actin served as loading control.

Cytokine response to TLR9 stimulation using synthetic TLR9 ligands in wild type and TLR9-engineered HEK293 cells

Signaling via TLRs ultimately leads to the expression of pro-inflammatory cytokines, chemokines and anti-microbial effector molecules (Kawai & Akira, 2011). Cytokines such as IL-6 and TNF-α can be used to monitor the response to TLR stimulation in HEK293 cells (Assaf *et al.*, 2009; Razonable *et al.*, 2005). We investigated the functionality of TLR9 signaling in both cell lines upon stimulation of TLR9 with synthetic CpG oligodeoxynucleotides (CpGs). We used two different classes of CpGs, class A and B, for activation of TLR9 signaling, to determine the most effective stimuli since both classes can evoke distinct reactions (Krieg, 2002; Krug *et al.*, 2001; Rothenfusser *et al.*, 2001). We measured the changes in *IL-6* and *TNF-α* gene expression in response to TLR9 triggering by RT-qPCR and compare it to mock-stimulated cells.

Upon stimulation with CpG-B, 293-WT cells showed a 4.1-fold (±1.2) increased expression of *IL-6* compared to mock-stimulated cells after 4h of stimulation. Stimulation with CpG-A did not change *IL-6* expression significantly (Fig. 2A). TLR9 stimulation in 293-TLR9 cells did not lead to an *IL-6* response at all (Fig. 2B). In contrast, 293-WT cells did not show a *TNF-α* response to TLR9 stimulation (Fig. 2C) while 293-TLR9 cells showed a strong induction of 9.8-fold (±0.4) in *TNF-α* expression 2h post stimulation (Fig. 2D). The expression of *TNF-α* in 293-TLR9 reached a peak after 4h of stimulation with a 18.7-fold (±0.01) induction compared to mock-stimulated cells and was still 7.3-fold (±0.1) up-regulated 6h post stimulation (Fig. 2D). Taken together, both cell lines responded to TLR9 stimulation with CpG-B but not to CpG-A, while 293-WT cells showed increased *IL-6* expression 4h post stimulation and 293-TLR9 cells responded by strong up-regulation of

TNF-α gene expression. In conclusion, the stimulation with CpG-A is either without effect or we might not have chosen the proper readout to detect a response to CpG-A. Additionally, the *TNF-α* response of 293-TLR9 cells appears to be more reliable since it was shown that HEK293 cells du not respond to CpG-B stimulation by IL-6 secretion (Assaf *et al.*, 2009).

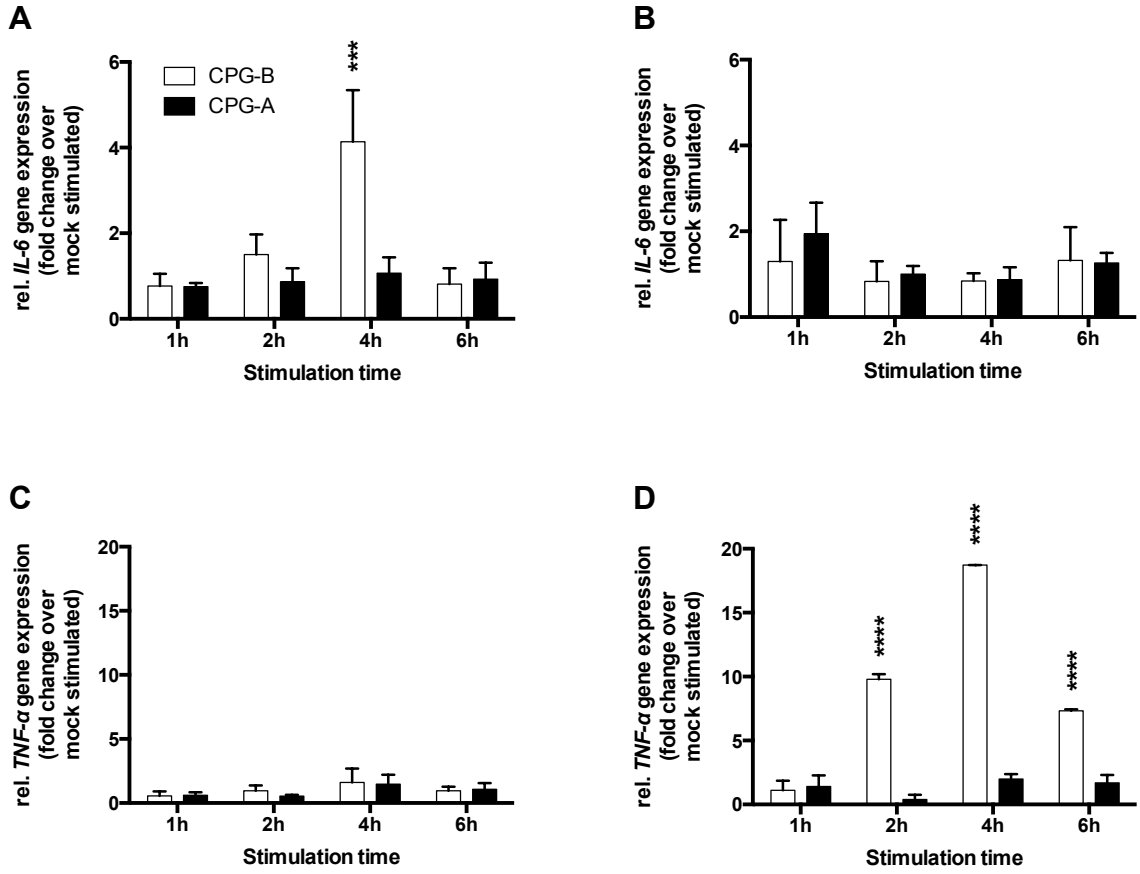

Figure 2: Cytokine response in HEK293 cell lines to TLR9 stimulation with synthetic TLR9 ligands. 293-WT (A; C) and 293-TLR9 (B; D) cells were stimulated with CpG-B (white) or CpG-A (black) and *IL-6* (A; B) and *TNF-α* (C; D) gene expression levels were determined as response to TLR9 stimulation by RT-qPCR relative to *HMBS*. Data is represented as fold change of the Mean ±SD to mock stimulated cells of at least 3 independent experiments; *** = $p<0.001$; **** = $p<0.0001$ (ordinary one-way ANOVA; Dunnett's multiple comparison test).

Continuous TLR9 stimulation and infection by EBV of wild type and TLR9-engineered epithelial cells

Next, we investigated the impact of TLR9 stimulation on the infection susceptibility and establishment of persisting infection in both cell lines. Therefore, we activated the signaling via TLR9 by stimulation with both types of CpG ODNs, class B and A, 2h prior to EBV inoculation and re-stimulated the cells 24h and 48h thereafter. We determined the amount of infected cells 72h p.i. by detection of GFP using flow cytometry.

293-WT cells showed in general about 3.5-fold higher infection frequencies as compared to 293-TLR9 cells. Infection frequencies of mock-stimulated 293-WT cells were 1.34% (±0.12) while CpG-B and CpG-A stimulation resulted in infection frequencies of 1.33% (±0.12) and 1.45% (±0.15), respectively. Mock-stimulated 293-TLR9 cells showed infection frequencies of 0.32% (±0.00), with CpG-B stimulation of 0.38% (±0.02) and with CpG-A stimulation of 0.37% (±0.09). As shown in Figure 3 there was no difference due to TLR9 stimulation in 293-WT (Fig. 3A) and in 293-TLR9 (Fig. 3B) cells compared to the corresponding mock-stimulated cells. Taken together, we did not observe any impact of TLR9 stimulation on the infection frequencies of 293-WT and 293-TLR9 cells, nor on the persistence of EBV. The lower infection frequencies of 293-TLR9 cells suggest that a clone with reduced susceptibility to EBV infection was selected during the establishment of the cell line.

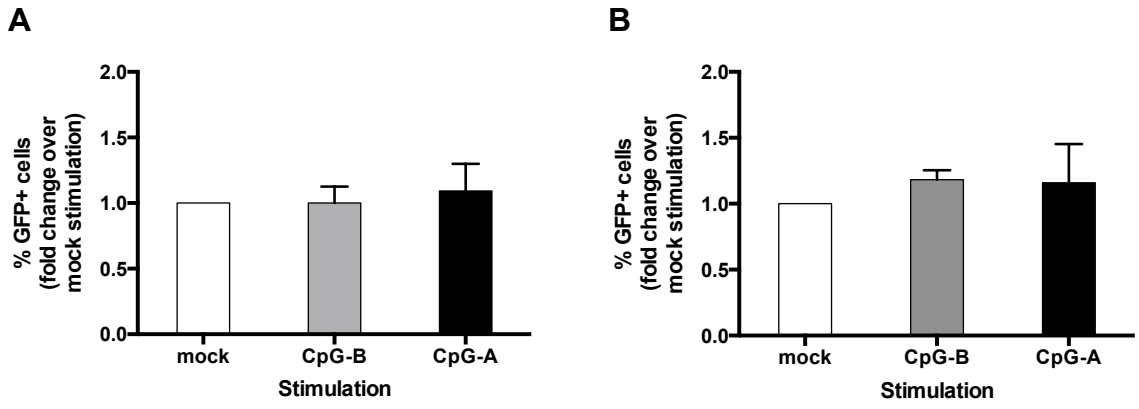

Figure 3: Infection frequencies of HEK293 cell lines upon direct infection by EBV under continuous TLR9 stimulation with synthetic TLR9 ligands. 293-WT (A) and 293-TLR9 (B) cells were stimulated with CpG-B (grey) or CpG-A (black) and subjected to direct infection by EBV. Infection frequencies were determined 72 h p.i. by flow cytometry within the living cell (7-AAD negative) population and shown as fold change over mock stimulated cells (white). Data is represented as Mean ±SD of 3 independent experiments.

EBV gene expression in wild type and TLR9-engineered epithelial cells upon direct infection by EBV with continuous TLR9 stimulation

Knowing that TLR9 stimulation can drive EBV into latency and inhibit the switch from latent to lytic replication (Ladell *et al.*, 2007; Zauner *et al.*, 2010) as well as that activation of TLR2 can induce lytic EBV replication (Ueda *et al.*, 2014) we went on to determine the impact of TLR9 stimulation on EBV gene expression in epithelial cell lines. We determined the expression of the EBV non-coding RNA EBER1, of three latency-associated genes *EBNA1*, *EBNA2* and *LMP1* and of two genes associated with lytic EBV

replication, *BZLF1* and *BXLF2*, in 293-WT (Fig. 4) and in 293-TLR9 (Fig. 5) cells upon direct EBV infection and continuous TLR9 stimulation with either CpG-B or CpG-A, as mentioned above, in the epithelial model cell lines 293-WT and 293-TLR9.

In general, consistent with the low infection frequencies we observed low expression of all EBV genes including EBER1. In 293-WT cells, the expression of EBER1 was more or less constant although it was decreased in mock- and CpG-B-treated samples 48h p.i. (Fig. 4A). The gene expression of *EBNA1*, *EBNA2* and *BXLF2* was decreasing over time in 293-WT cells (Fig. 4B, C and F), independently of the treatment, while CpG-A treated cells showed a slower decrease of *EBNA2* and *BXLF2* gene expression. *LMP1* and *BZLF1* gene expression hardly reached the limit of detection indicated by the dotted horizontal line, but remained relatively constant (Fig. 4D and E). However, when we compared the expression levels to mock-stimulated 293-WT cells we did not detect any significant effect on EBV gene expression due to treatment with CpG-B and CpG-A, respectively. Taken together, these results indicate that neither treatment with CpG-B nor with CpG-A had an affect on EBV gene expression in 293-WT cells and thus did not promote EBV latency or EBV persistence.

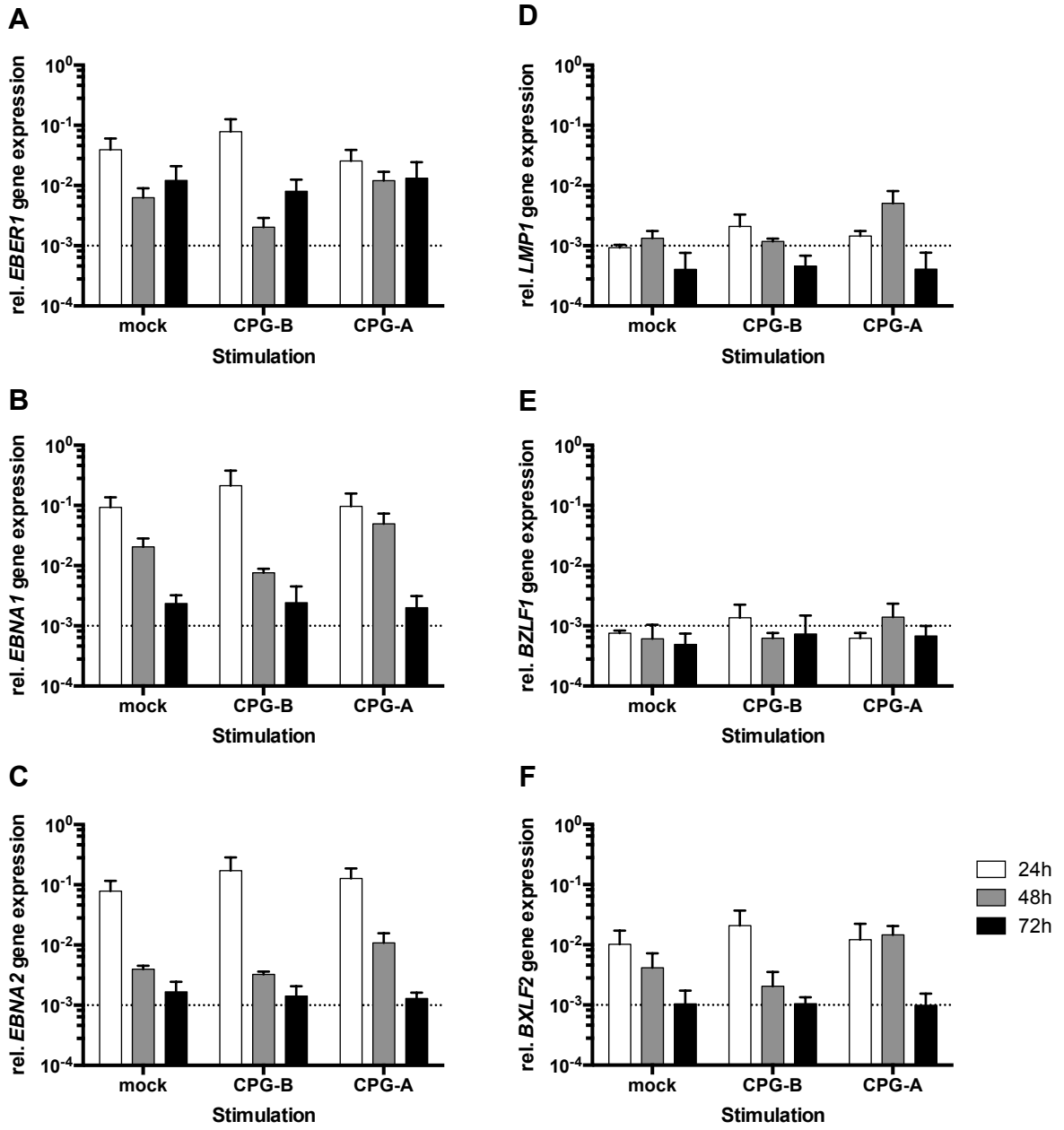

Figure 4: EBV gene expression in 293-WT cells upon direct infection by EBV under continuous TLR9 stimulation with CpG-B or CpG-A. Expression of EBER1 (A), *EBNA1* (B), *EBNA2* (C), *LMP1* (D), *BZLF1* (E) and *BXLF2* (F) was determined in mock-, CpG-B or CpG-A stimulated 293-WT cells by RT-qPCR relative to *HMBS*. Data is represented as Mean ±SD of 3 independent experiments and tested for statistical significance by unpaired t tests to mock-stimulated samples; dotted horizontal line indicates the limit of detection.

In 293-TLR9 cells we detected even lower gene expression levels as compared to 293-WT cells, which we expected due to the lower infection frequencies in these cells as mentioned above. Additionally, we had to exclude the gene expression data for *EBNA1* in 293-TLR9 from our analysis (Fig. 5B) these cells contain an EBNA1-based expression system, which did not allow us to investigate the gene expression of *EBNA1* from the viral

genome. The expression of EBER1 was slightly increased in mock and CpG-A stimulated 293-TLR9 cells 48h p.i. and was decreased 72h p.i., while EBER1 expression was not affected in CpG-B stimulated cells (Fig. 5A). However, compared to mock-stimulated cells the expression of EBER1 remained higher 72h p.i. in CpG-B and CpG-A treated cells. As seen in 293-WT cells, the gene expression of *EBNA2* and *BXLF2* in 293-TLR9 cells was decreasing over time in mock- and CpG-B stimulated cells, while CpG-A treated cells showed a slight increase in *EBNA2* and *BXLF2* gene expression 48h p.i. (Fig. 5C and F). Although the gene expression levels of *EBNA2* and *BXLF2* barely reached the limit of detection 72h p.i., they were again higher in CpG-B and CpG-A treated cells compared to mock-stimulated cells. *LMP1* and *BZLF1* were, as shown for 293-WT cells, again only weak or even not expressed at all in 293-TLR9 cells (Fig. 5D and E). Once again, we did not observe any significant effect on EBV gene expression upon treatment with CpG-B and CpG-A, respectively, in 293-TLR9 cells. Nevertheless, since the expression levels of EBER1, *EBNA2* and *BXLF2* were still higher 72h p.i. in CpG-B and CpG-A treated 293-TLR9 cells compared to mock-stimulated cells, these results might imply that TLR9 stimulation could contribute to EBV replication in epithelial cells.

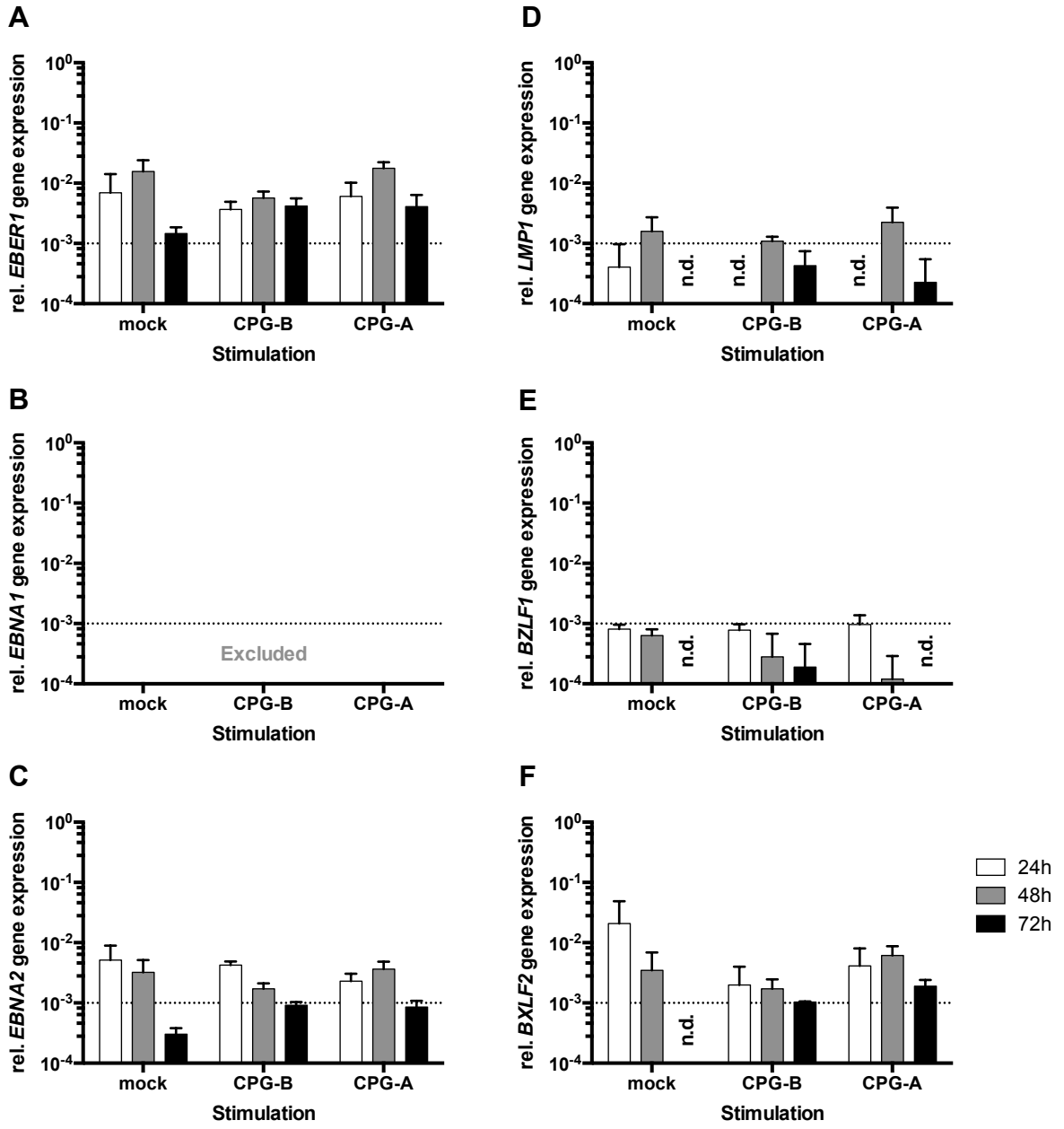

Figure 5: EBV gene expression in 293-TLR9 cells upon direct infection by EBV under continuous TLR9 stimulation with CpG-B or CpG-A. Expression of EBER1 (A), *EBNA1* (B), *EBNA2* (C), *LMP1* (D), *BZLF1* (E) and *BXLF2* (F) was determined in mock-, CpG-B or CpG-A stimulated 293-TLR9 cells by RT-qPCR relative to *HMBS*. Data is represented as Mean ±SD of 3 independent experiments and tested for statistical significance by unpaired t tests to mock-stimulated samples; n.d. = not detected; dotted horizontal line indicates the limit of detection.

IX.2.5 Discussion

EBV is transmitted via saliva and enters the host via the oral mucosal epithelium that is *per se* constantly challenged by various microbes. In this study we employed an epithelial cell model to test the influence of TLR9 stimulation on the EBV infection in epithelial cells.

We found that 1) individual cell lines show a distinct TLR expression pattern; 2) TLR9-engineered epithelial cells respond to TLR9 stimulation with CpG-B by up-regulation of *TNF-α* gene expression; 3) TLR9 stimulation with CpG-B and CpG-A has no impact on infection frequencies of epithelial model cells and does not alter EBV gene expression significantly. Our results indicate that the employed epithelial model cell lines 293-WT and 293-TLR9 may not be physiological models to study the impact of TLR signaling on the infection in epithelial cells by EBV, but suggest a contribution of TLR activation to reactivation of EBV from latency in epithelial cells.

Comparing the *TLR* gene expression pattern in 293-WT and 293-TLR9 cells we found obvious differences between distinct cell lines. Both cell lines should originate from the same HEK293 cells described by Graham *et al.* (Graham *et al.*, 1977). Wild type HEK293 cells possess functional signaling upon activation of endogenous TLR5 (Huang *et al.*, 2009; Simon & Samuel, 2007; Smith *et al.*, 2003) but they do not express TLR2, 4 (Erridge *et al.*, 2007; Latz *et al.*, 2002) and TLR6 at protein level (Erridge *et al.*, 2007). It was as well described that low levels of TLR9 mRNA can be detected but without functional signaling via TLR9 (Assaf *et al.*, 2009). Additionally, Assaf *et al.* detected *TLR9* gene expression levels in TLR9-transfected HEK293 cells comparable to various B-cell lines (Assaf *et al.*, 2009). Since the employed primer/probe set, used to amplify a specific fragment within the 5' untranslated region of the TLR9 gene, is specific for endogenous *TLR9* and because the expression construct for TLR9 within the 293-TLR9 cells contains only the coding region for TLR9, we could not detect ectopically expressed *TLR9*. The strong *TLR4* gene expression in 293-TLR9 cells was unexpected. Functional signaling via TLR4 requires co-expression of MD-2 and CD14 (Latz *et al.*, 2002). However, since wild type HEK293 cells are usually deficient for both molecules we did not further investigate whether the here measured *TLR4* expression exhibited functional signaling. The cell line 293-TLR9 was selected from single cell clones, showing the best response to TLR9 stimulation, without regard to the overall TLR expression pattern (personal communication by the manufacturer). Therefore, we conclude that the altered *TLR* gene expression pattern in 293-TLR9 cells is a result of the clonal selection procedure *in vitro*.

We determined *IL-6* and *TNF-α* gene expression as response to TLR9 stimulation with two distinct classes of synthetic CpGs. To our surprise, 293-WT cells responded to treatment with CpG-B with slight up-regulation of *IL-6*, while 293-TLR9 cells showed a strong *TNF-α* response to CpG-B stimulation. Notably, apart from NF-κB activation and induction of

proliferation in TLR9-transfected HEK293 cells, Assaf and colleagues did not observe any difference such as increased IL-6 or TNF-α secretion or up-regulation of surface markers in control and TLR9-transfected HEK293 cells upon TLR9 stimulation with another class B CpG (Assaf *et al.*, 2009). Nevertheless, the strong *TNF-α* response we observed in 293-TLR9 cells appears to be more of biological relevance since these cells were shown to be capable of NF-κB activation upon TLR9 stimulation (Assaf *et al.*, 2009), which would lead to *TNF-α* expression. To fully confirm functional TLR9 signaling we would additionally need to assess secretion of these cytokines. However, as mentioned above, Assaf et al. already demonstrated that control and TLR9-engineeried are not capable to secrete IL-6, TNF- α and IL-10 upon TLR9 activation. Thus, additionally questioning the biological relevance of these model cell lines with respect to the epithelium and the effect of TLR activation on the EBV infection in epithelial cells.

The stimulation with CpG-A had no effect on cytokine expression in 293-WT and 293-TLR9 cells. Following their distinct structural and biological characteristics, three classes of CpG ODNs have been defined (Hartmann *et al.*, 2003; Marshall *et al.*, 2003; Vollmer *et al.*, 2004). B-class CpGs strongly activate B cells and have only little effects on induction of natural killer (NK) cell activity and interferon-alpha (IFN-α) secretion (Krieg, 2002). On the other hand, CpG class A molecules induce a strong NK cell activation and IFN-α secretion from plasmacytoid dendritic (pDC) cells, but only weakly stimulate B cells (Krug *et al.*, 2001; Rothenfusser *et al.*, 2001). CpG ODNs from class C combine the functional properties from class A and B (Hartmann *et al.*, 2003; Vollmer *et al.*, 2004). Although all classes of CpG ODNs are sensed by the same TLR9 receptor and CpG-B molecules were frequently used in studies especially with airway and intestinal epithelial cells, we might have missed the activation upon CpG-A stimulation of the tested cell lines probably due to an inappropriate readout.

We did not observe any significant effect on the infection frequencies or on EBV gene expression in 293-WT or 293-TLR9 cells due TLR9 stimulation. Since we recently demonstrated that TLR9 stimulation prevents lytic EBV infection upon primary infection of B cells and inhibits lytic reactivation in EBV positive BL cells (Ladell *et al.*, 2007; Zauner *et al.*, 2010) we hypothesized that TLR9 stimulation might lead to or support the establishment of a latent EBV infection as well in epithelial cells. Given that *in vitro* EBV is rapidly lost from epithelial cells (Glaser *et al.*, 1989; Shannon-Lowe *et al.*, 2009; Wu *et al.*, 2003; Yao *et al.*, 1990) and that TLR9 stimulation might lead to the establishment of a latent EBV infection

in epithelial cells, we expected to be able to measure higher infection frequencies in 293-TLR9 cells upon TLR9 stimulation. This was not the case, however we detected slightly increased expression of EBER1 and *EBNA2* and *BXLF2* in 293-TLR9 cells upon CpG-B and A treatment compared to mock stimulated cells 72h p.i.. Taken into account that we achieved only very low infection frequencies (about 0.5% infected 293-TLR9 cells), resulting in very low EBV gene expression levels, and that the expression of the late lytic gene *BXLF2* was as well increased we speculate that TLR9 stimulation in epithelial cells might contribute to lytic EBV replication. This is compatible with the finding that TLR2 triggering in EBV positive LCLs results in lytic EBV replication (Ueda *et al.*, 2014).

HEK293 cells and especially the TLR9-engineered HEK293 cells used in this study have some limitations. First, these model cell lines show only little relation to epithelial cells within the pharyngeal cavities regarding their TLR expression pattern. Especially, TLR2, 3, 4, which are not or only weak expressed in HEK293 cells, seem to play a more important role in primary epithelial cells and cell lines from the pharyngeal cavities than TLR9 (Claeys *et al.*, 2003; Lange *et al.*, 2009b; Swaminathan *et al.*, 2013; Tezera *et al.*, 2011; Vandermeer *et al.*, 2004; Yamada *et al.*, 2011). Second, we could achieve only low infection frequencies in 293-TLR9 cells probably because of the selection of a cell clone with reduced susceptibility to EBV infection during the establishment of the cell line. Third, 293-TLR9 cells were established by using an EBV based expression plasmid and therefore constitutively express EBNA1 that is essential for episomal plasmid and EBV genome maintenance (Frappier, 2012) and will influence EBV gene expression within these cells. It results that the TLR9-engineered HEK293 cell line itself might not provide a suitable model to study the impact of TLR9 or in general TLR signaling on the infection of epithelial cells by EBV. The establishment of primary epithelial cell cultures or established cell lines from the pharyngeal cavities might provide a considerably more physiological model, thus facilitating the study of TLR signaling and its impact on the EBV infection in epithelial cells especially with regard to TLRs apart from TLR9.

Taken together, we show here that activation of TLR9 signaling by synthetic ligands in the model cell lines 293-WT and 293-TLR9 does not have a significant impact on EBV infection. This does not exclude that EBV might actually benefit from TLR activation within the pharyngeal cavities regarding virus transmission. Pharyngeal epithelial cells are in general believed to serve as amplifier and to be the source of progeny virus for shedding (Hadinoto *et al.*, 2009; Perera *et al.*, 2010). Our observation of a slightly increased EBV gene expression in

293-TLR9 cells upon CpG stimulation, especially of the late lytic gene *BXLF2*, without increased infection frequencies, might potentially reflect an ongoing EBV replication. This leads us to the hypothesis that activation of TLRs in pharyngeal epithelial cells might serve as alarm signal for EBV, triggering lytic replication and therefore ensuring virus transmission and spreading to the next naïve host, supported by the recent finding that GAS colonizes patients shed higher numbers of EBV particles into saliva (Ueda *et al.*, 2014).

IX.2.6 Acknowledgment

This work was supported by the Swiss National Science Foundation (grant 310030_135028 to D. N.) and the Cancer League of the Canton Zurich (to D. N.).

IX.2.7 References

Ariza, M.-E., Glaser, R., Kaumaya, P. T. P., Jones, C. & Williams, M. V. (2009). The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. *J Immunol* **182**, 851–9.

Assaf, A., Esteves, H., Curnow, S. J. & Browning, M. J. (2009). A threshold level of TLR9 mRNA predicts cellular responsiveness to CpG-ODN in haematological and non-haematological tumour cell lines. *Cell Immunol* **259**, 90–9. Elsevier Inc.

Bonanomi, A., Kojic, D., Giger, B., Rickenbach, Z., Jean-Richard-Dit-Bressel, L., Berger, C., Niggli, F. K. & Nadal, D. (2003). Quantitative cytokine gene expression in human tonsils at excision and during histoculture assessed by standardized and calibrated real-time PCR and novel data processing. *J Immunol Methods* **283**, 27–43.

Claeys, S., de Belder, T., Holtappels, G., Gevaert, P., Verhasselt, B., van Cauwenberge, P. & Bachert, C. (2003). Human beta-defensins and toll-like receptors in the upper airway. *Allergy* **58**, 748–53.

Delecluse, H. J., Hilsendegen, T., Pich, D., Zeidler, R. & Hammerschmidt, W. (1998). Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc Natl Acad Sci U S A* **95**, 8245–50.

Diamond, G., Beckloff, N. & Ryan, L. K. (2008). Host defense peptides in the oral cavity and the lung: similarities and differences. *J Dent Res* **87**, 915–27.

Dirmeier, U., Neuhierl, B., Kilger, E., Reisbach, G., Sandberg, M. L. & Hammerschmidt, W. (2003). Latent membrane protein 1 is critical for efficient growth transformation of human B cells by epstein-barr virus. *Cancer Res* **63**, 2982–9.

Dorner, M., Zucol, F., Berger, C., Byland, R., Melroe, G. T., Bernasconi, M., Speck, R. F. & Nadal, D. (2008). Distinct ex vivo susceptibility of B-cell subsets to epstein-barr virus infection according to differentiation status and tissue origin. *J Virol* **82**, 4400–12.

Erridge, C., Spickett, C. M. & Webb, D. J. (2007). Non-enterobacterial endotoxins stimulate human coronary artery but not venous endothelial cell activation via Toll-like receptor 2. *Cardiovasc Res* **73**, 181–9.

Feederle, R., Neuhierl, B., Bannert, H., Geletneky, K., Shannon-Lowe, C. & Delecluse, H.-J. (2007). Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection. *Int J Cancer* **121**, 588–94.

Feller, L., Altini, M., Khammissa, R. a G., Chandran, R., Bouckaert, M. & Lemmer, J. (2013). Oral mucosal immunity. *Oral Surg Oral Med Oral Pathol Oral Radiol* **116**, 576–83. Elsevier Inc.

Fiola, S., Gosselin, D., Takada, K. & Gosselin, J. (2010). TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells. *J Immunol* **185**, 3620–31.

Frappier, L. (2012). The Epstein-Barr Virus EBNA1 Protein. *Scientifica (Cairo)* **2012**, 438204.

Gaudreault, E., Fiola, S., Olivier, M. & Gosselin, J. (2007). Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2. *J Virol* **81**, 8016–24.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59–74.

Griffin, B. D., Verweij, M. C. & Wiertz, E. J. H. J. (2010). Herpesviruses and immunity: the art of evasion. *Vet Microbiol* **143**, 89–100. Elsevier B.V.

Hadinoto, V., Shapiro, M., Sun, C. C. & Thorley-Lawson, D. a. **(2009).** The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. *PLoS Pathog* **5**, e1000496.

Hartmann, G., Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S. & Endres, S. (2003). Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur J Immunol* **33**, 1633–41.

Hislop, A. D., Taylor, G. S., Sauce, D. & Rickinson, A. B. (2007). Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* **25**, 587–617.

Hornef, M. W. & Bogdan, C. (2005). The role of epithelial Toll-like receptor expression in host defense and microbial tolerance. *J Endotoxin Res* **11**, 124–8.

Huang, L.-Y., Dumontelle, J. L., Zolodz, M., Deora, A., Mozier, N. M. & Golding, B. (2009). Use of toll-like receptor assays to detect and identify microbial contaminants in biological products. *J Clin Microbiol* **47**, 3427–34.

Iwakiri, D., Zhou, L., Samanta, M., Matsumoto, M., Ebihara, T., Seya, T., Imai, S., Fujieda, M., Kawa, K. & Takada, K. (2009). Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from toll-like receptor 3. *J Exp Med*. **206**:2091-9

Kawai, T. & Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–50. Elsevier Inc.

Kieff, E. D. & Rickinson, A. B. (2007). Epstein-Barr Virus and Its Replication. In *Fields Virol*, 5th edn., pp. 2603–2654. Edited by D. M. Knipe & P. M. Howley. Lippincott Williams & Wilkins.

Krieg, A. M. (2002). CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* **20**, 709–60.

Krug, a, Rothenfusser, S., Hornung, V., Jahrsdörfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, a M. & Hartmann, G. (2001). Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* **31**, 2154–63.

Ladell, K., Dorner, M., Zauner, L., Berger, C., Zucol, F., Bernasconi, M., Niggli, F. K., Speck, R. F. & Nadal, D. (2007). Immune activation suppresses initiation of lytic Epstein-Barr virus infection. *Cell Microbiol* **9**, 2055–69.

Lange, M. J., Lasiter, J. C. & Misfeldt, M. L. (2009a). Toll-like receptors in tonsillar epithelial cells. *Int J Pediatr Otorhinolaryngol* **73**, 613–21.

Lange, M. J., Lasiter, J. C. & Misfeldt, M. L. (2009b). Toll-like receptors in tonsillar epithelial cells. *Int J Pediatr Otorhinolaryngol* **73**, 613–21.

Latz, E., Visintin, A., Lien, E., Fitzgerald, K. a, Monks, B. G., Kurt-Jones, E. a, Golenbock, D. T. & Espevik, T. (2002). Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem* **277**, 47834–43.

Marshall, J. D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R. L. & Van Nest, G. (2003). Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol* **73**, 781–92.

Martin, H. J., Lee, J. M., Walls, D. & Hayward, S. D. (2007). Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. *J Virol* **81**, 9748–58.

Neuhierl, B., Feederle, R., Hammerschmidt, W. & Delecluse, H. J. (2002). Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc Natl Acad Sci U S A* **99**, 15036–41.

Perera, R. A. P. M., Samaranayake, L. P. & Tsang, C. S. P. (2010). Shedding dynamics of Epstein-Barr virus: A type 1 carcinogen. *Arch Oral Biol* **55**, 639–47. Elsevier Ltd.

Razonable, R. R., Henault, M., Lee, L. N., Laethem, C., Johnston, P. A., Watson, H. L. & Paya, C. V. (2005). Secretion of proinflammatory cytokines and chemokines during amphotericin B exposure is mediated by coactivation of toll-like receptors 1 and 2. *Antimicrob Agents Chemother* **49**, 1617–21.

Rickinson, A. B. & Kieff, E. D. (2007). Epstein-Barr Virus. In *Fields Virol*, 5th edn., pp. 2656–2700. Edited by D. M. Knipe & P. M. Howley. Lippincott Williams & Wilkins.

Rothenfusser, S., Hornung, V., Krug, A., Towarowski, A., Krieg, A. M., Endres, S. & Hartmann, G. (2001). Distinct CpG oligonucleotide sequences activate human gamma delta T cells via interferon-alpha/-beta. *Eur J Immunol* **31**, 3525–34.

Sha, Q., Truong-Tran, A. Q., Plitt, J. R., Beck, L. a & Schleimer, R. P. (2004). Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* **31**, 358–64.

Simon, R. & Samuel, C. E. (2007). Activation of NF-kappaB-dependent gene expression by Salmonella flagellins FliC and FljB. *Biochem Biophys Res Commun* **355**, 280–5.

Smith, M. F., Mitchell, A., Li, G., Ding, S., Fitzmaurice, A. M., Ryan, K., Crowe, S. & Goldberg, J. B. (2003). Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* **278**, 32552–60.

Speck, S. H. & Ganem, D. (2010). Viral latency and its regulation: lessons from the gamma-herpesviruses. *Cell Host Microbe* **8**, 100–15. Elsevier Inc.

Swaminathan, V., Prakasam, S., Puri, V. & Srinivasan, M. (2013). Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis. *J Periodontal Res* **48**, 757–65.

Taylor, G. S. & Blackbourn, D. J. (2011). Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV. *Cancer Lett* **305**, 263–78. Elsevier Ireland Ltd.

Tezera, L. B., Hampton, J., Jackson, S. K. & Davenport, V. (2011). Neisseria lactamica attenuates TLR-1/2-induced cytokine responses in nasopharyngeal epithelial cells using PPAR-γ. *Cell Microbiol* **13**, 554–68.

Ueda, S., Uchiyama, S., Azzi, T., Gysin, C., Berger, C., Bernasconi, M., Harabuchi, Y., Zinkernagel, A. S. & Nadal, D. (2014). Oropharyngeal group a streptococcal colonization disrupts latent epstein-barr virus infection. *J Infect Dis* **209**, 255–64.

Vandermeer, J., Sha, Q., Lane, A. P. & Schleimer, R. P. (2004). Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and toll-like receptors. *Arch Otolaryngol Head Neck Surg* **130**, 1374–80.

Vérillaud, B., Gressette, M., Morel, Y., Paturel, C., Herman, P., Lo, K. W., Tsao, S. W., Wassef, M., Jimenez-Pailhes, A.-S. & Busson, P. (2012). Toll-like receptor 3 in Epstein-Barr virus-associated nasopharyngeal carcinomas: consistent expression and cytotoxic effects of its synthetic ligand poly(A:U) combined to a Smac-mimetic. *Infect Agent Cancer* **7**, 36.

Vollmer, J., Weeratna, R., Payette, P., Jurk, M., Schetter, C., Laucht, M., Wader, T., Tluk, S., Liu, M. & other authors. (2004). Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol* **34**, 251–62.

Wang, J., Shao, Y., Bennett, T. a, Shankar, R. a, Wightman, P. D. & Reddy, L. G. (2006). The functional effects of physical interactions among Toll-like receptors 7, 8, and 9. *J Biol Chem* **281**, 37427–34.

West, J. a, Gregory, S. M. & Damania, B. (2012). Toll-like receptor sensing of human herpesvirus infection. *Front Cell Infect Microbiol* **2**, 122.

Yamada, T., Jiang, X., Kubo, S., Sakashita, M., Narita, N., Yamamoto, H., Sunaga, H. & Fujieda, S. (2011). B type CpG-DNA suppresses poly(I:C)-induced BLyS expression and production in human tonsillar fibroblasts. *Clin Immunol* **141**, 365–71. Elsevier Inc.

Zauner, L., Melroe, G. T., Sigrist, J. a, Rechsteiner, M. P., Dorner, M., Arnold, M., Berger, C., Bernasconi, M., Schaefer, B. W. & other authors. (2010). TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. *Oncogene* **29**, 4588–98. Nature Publishing Group.

X ACKNOWLEDGMENTS

A doctoral thesis is so much more than just the sum of all the experiments performed. It would not have been possible to reach this point without the great support of many people. Therefore I would like to thank all the people who guided and supported me during my time as Ph.D. student, in particular:

Prof. Dr. David Nadal

I am deeply indebted to you for your constant support and motivation, for your steady guidance and for providing me with the opportunity to participate in this challenging research project. I appreciate that your door was always open for discussion.

Dr. Michele Bernasconi

Thank you so much for your support and patience. I appreciated your technical and scientific guidance as much as I enjoyed any off-topic discussion we had and last but not least thank you for always having a kind word when I needed some encouragement.

Prof. Dr. Urs F. Greber, Prof. Dr. Michael O. Hottiger, Prof. Dr. Roberto F. Speck and Dr. Bernd Wollscheid

Thank you for being members of my thesis committee, for taking your time to judge my yearly progress and your stimulating suggestions for my project.

Former and present members of the Infectiology group: Tarik Azzi, Andrea Burch Tamara Bösch, Simone Bürgler, Florian Haas, Marc Jordi, Patricia Krukowski, Anna Lünemann, Jeannine Marty, Vanessa Mordasini, Monika Murat, Corinne Peng, Jürg Sigrist, Seigo Ueda, Kazuma Yamauchi and Ludwig Zauner

It was a great pleasure for me to be part of this fantastic group. Thank you so much for your support and for sharing all these happy and sometimes as well frustrating moments I experienced during the last years.

Andrina Schumacher

Thank you for being the best master student I could imagine. It was a wonderful challenge for me to guide you through your master thesis and I probably learned as much from you as you did from me. I really enjoyed working with you!

All the present and former members I got to know of the oncology and leukemia groups

Thank you very for the time we spend together, working, discussing, chatting, laughing and celebrating. It is because of all of you that working and spending my time at the AFS1 was (almost) always a joy.

My parents Renate & Heinz and my brother Peter

I am forever indebted to you. I wouldn't be the person I'm today without your constant and unconditional support throughout my entire life. You are the best family I can imagine and I will always be grateful.

Nadine

Thank you for being me a support when I needed it and for tolerating all my moods during my PhD studies. From the moment I've seen you (in the probably most boring course I can imagine), I knew that you are something special and I'm extremely happy that you and our big joint experiment became part my life.

XI CURRICULUM VITAE

Name:	**Jürgen RAC**
Date of birth:	**February 20th, 1982**
Place of birth:	**Giengen a.d. Brenz, Baden-Württemberg, Germany**
Nationality:	**German**

Education:

since 01.01.2010	**University of Zurich/University Children's Hospital of Zurich**
	PhD thesis (Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital of Zurich, University of Zurich, Zurich, Switzerland)
08/2009	**University of Stuttgart**
	Finished the Study of technical Biology with the overall grade 1.4 (Diplom-Biologe (technisch orientiert))
09/2008 – 08/2009	**University of Stuttgart**
	Diploma thesis in the group of Prof. Dr. Jens Kurreck (Institute for Industrial Genetics)
	Title: "Establishment of a replication system for the torque teno virus tth25" (grade 1.0)
07/2006 – 08/2008	**University of Stuttgart (Final Exams)**
	Industrial Genetics (Prof. Dr. Jens Kurreck and Prof. Dr. Franziska Wollnik; HF; grade 1.0)
	Microbiology (Prof. Dr. Andreas Stolz; NF; grade 1.0)
	Technical Biochemistry (Prof. Dr. Rolf D. Schmid; NF; grade 1.7)
	Biochemistry (Prof. Dr. Dieter H. Wolf; WF; grade 2.0)
10/2002 – 08/2009	**University of Stuttgart**
	Study of technical Biology at the University of Stuttgart (Baden-Württemberg, Germany)
07/2001 – 08/2002	**Civilian Service**
	("Sozialpsychiatrischer Wohnverbund", Heidenheim a.d. Brenz, Baden-Württemberg, Germany)
08/1992 – 06/2001	**"Allgemeine Hochschulreife"**
	(Werkgymnasium Heidenheim, Heidenheim a.d. Brenz, Baden-Württemberg, Germany)